Targeted Proteomics Applied in Clinical Diagnostics and Doping Analysis

- Immuno-MS based Determination of Human Chorionic Gonadotropin

Thesis for the degree of Philosophiae Doctor

by

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Oslo, December 2012

Hanne Lund

LIST OF PAPERS

This thesis is based on the following papers which will be referred to by their roman numeral in the text:

I **H. Lund**, S.B. Torsetnes, E. Paus, K. Nustad, L. Reubsaet, T.G. Halvorsen *"Exploring the complementary selectivity of immunocapture and MS detection for the differentiation between hCG isoforms in clinically relevant samples"*, J. Prot. Res, (2009)

II **H. Lund**, K. Løvsletten, E. Paus, T.G. Halvorsen, L. Reubsaet *"Immuno-MS based targeted proteomics: Highly specific, sensitive, and reproducible human chorionic gonadotropin determination for clinical diagnostics and doping analysis"*, Anal. Chem. (2012)

III **H. Lund**, A.H. Snilsberg, E. Paus, T.G. Halvorsen, P. Hemmersbach, L. Reubsaet *"Sports drug testing using immuno-MS: clinical study comprising administration of human chorionic gonadotropin to males"* Anal. Bioanal. Chem. (2012)

IV **H. Lund,** A.H. Snilsberg, T.G. Halvorsen, P. Hemmersbach, L. Reubsaet *"Comparison of newly developed immuno-MS method with existing DELFIA immunoassay for human chorionic gonadotropin determination in doping analysis"* Bioanalysis (2013)

V **H. Lund**, E. Paus, P. Berger, U.H. Stenman, T. Torcellini, T.G. Halvorsen, L. Reubsaet *"Epitope Analysis and Detection of human chorionic gonadotropin (hCG) Variants by Monoclonal Antibodies and Mass Spectrometry"* To be submitted to Tumor Biology

Papers not included in the dissertation:

VI M. Balchen, **H. Lund**, L. Reubsaet, S. Pedersen-Bjergaard *"Fast, selective, and sensitive analysis of low-abundance peptides in human plasma by electromembrane extraction"*, Anal. Chim. Acta (2012)

VII S.B. Torsetnes, S.G. Løvbak, **H. Lund**, M.S. Nordlund, E. Paus, T.G. Halvorsen, L. Reubsaet *"Immunocapture LC-MS/MS for selective quantification and differentiations of the isozymes of the biomarker NSE"* Submitted for publication

ABBREVATIONS

ABC	Ammonium Bicarbonate
AQUA	Absolute Quantification Peptides
BLAST	Basic Local Alignment Search Tool
BSA	Bovine Serum Albumin
CID	Collision Induced Dissociation
CTP	Carboxy-terminal peptide
ELISA	Enzyme-Linked Immunosorbent Assay
EMEA	European Medicines Agency
ESI	Electrospray Ionization
FSH	Follicle-Stimulating Hormone
HCl	Hydrochloric Acid
HCOOH	Formic Acid
HPLC	High-Performance Liquid Chromatography
IS	Internal Standard
IEF	Isoelectric focusing
LC	Liquid Chromatography
LOD	Limit of Detection
LH	Luteinizing Hormone
LLOQ	Lower Limit of Quantification
MALDI	Matrix-Assisted Laser Desorption Ionization
MeCN	Acetonitirile
MRM	Multiple Reaction Monitoring
MS	Mass Spectrometry
MS/MS	Tandem Mass Spectrometry
m/z	Mass-to-charge Ratio
NBCI	National Center for Biotechnology Information
NSE	Neuron-Specific Enolase
QqQ	Triple Quadrupole Detector
ProGRP	Progastrin Releasing Peptide
PSAQ	Protein Standards for Absolute Quantification

r	Correlation Coefficient
R	Recovery
RP-HPLC	Reversed-Phase High-Performance Liquid Chromatography
RSD	Relative Standard Deviation
SID	Stable Isotope Dilution
SIM	Selected Ion Monitoring
S/N	Signal-to-Noise
SQ	Single Quadrupole
SPE	Solid Phase Extraction
SRM	Selected Reaction Monitoring
TSH	Thyroid-Stimulating Hormone
TFA	Trifluoroacetic Acid
UniProtKB	UniProt Knowledgebase
WADA	World Anti-Doping Agency

ABSTRACT

The principal objective of this thesis was to develop a liquid chromatography (LC) mass spectrometry (MS) based method using the targeted proteomics approach to determine low abundance protein biomarkers in complex matrixes. To enable adequate sensitivity of the method, immunoaffinity extraction was intended as sample preparation strategy preceding the LC-MS analysis. Furthermore, the developed method's application in clinically relevant scenarios was shown. For this purpose the human chorionic gonadotropin (hCG) family of macromolecules was chosen, as this diverse biomarker group is currently utilized in both pregnancy and cancer diagnostics, in addition to being a sports drug exploited for doping purposes.

In **Paper I** the proof of principle for the immuno-MS methodology was demonstrated. First, unique signature peptides representing the respective hCG proteins were established theoretically, using protein data bases and search algorithms ensuring the specificity of each peptide. Furthermore, following enzymatic cleavage of the hCG proteins into peptides using trypsin, the experimentally obtained LC-MS analysis enabled the multiplexed separation and detection of various hCG proteins in one single run, using a single quadrupole (SQ) detector in the selected ion monitoring (SIM) mode. Thereafter, specific immunoaffinity extraction of the target hCG proteins using a monoclonal antibody immobilized to the walls 96 wells microtiter plates followed by in-well tryptic digestion, a solid phase extraction (SPE) clean-up step, and LC-MS analysis demonstrated the proof of principle for combining the complementary techniques of immunoaffinity extraction and mass spectrometric detection. This allowed the identification of hCG from complex matrixes such as serum and urine. The developed immuno-MS method was finally used for the analysis of authentic cancer patient serum samples, and urine samples from a pregnant woman. Qualitative hCG differentiation of various hCG forms in these samples indicated the potential of the method to provide complementary diagnostic information to that derived from the conventional immunoassays.

In **Paper II**, quantitative validation of the method was described, along with its potential for use in clinical settings. First, the preliminary LC-MS analysis developed on a SQ detector was transferred to a triple quadrupole (QqQ) detector to enable selective, specific and sensitive selected reaction monitoring (SRM) detection of the hCG signature peptides. Furthermore, the antibodies used for immunocapture were transferred to magnetic beads for the selective

extraction of target molecules from large sample volumes. This resulted in substantial improvement of the methods sensitivity and specificity. Subsequently, validation of the method was performed according to key elements and recommendations defined by the European Medicines Agency in *Guideline on Validation of Bioanalytical Methods*, demonstrating robust, reproducible and accurate quantification of hCG from serum and urine, with satisfying limit of detection (LOD) of 5 IU/L for serum and 2 IU/L for urine. The main hCG variant responsible for the biological activity associated with the hCG molecules was quantified, whereas the other hCG degradation variants were qualitatively detected and differentiated. The clinical potential of the method was tested and resulted in quantitative measurements of hCG in cancer patients serum samples, and of hCG in urine and serum samples from pregnant women.

Besides it quantitative ability, the developed method also allowed the evaluation of antibody selectivity, thus showing a complementary utility for the developed method in clinical diagnostics (**Paper V)**. This was done since defined antibody selectivity is crucial in immunoassays to ensure accurate detection of target compounds whilst discriminating other compounds. Defined hCG standards were extracted using 30 different anti-hCG antibodies, and analyzed using the tailored hCG LC-MS/MS detection. The obtained results were further compared to those generated by an hCG radioimmunoassay performed in a parallel study, and the antibodies were classified in groups according to epitope recognition. The purity of the hCG standards was evaluated as well.

To investigate the method's applicability in doping analysis a clinical study comprising the administration of one out of two hCG containing pharmaceutical to 24 males was conducted (**Paper III**). Serum and urine samples were collected prior to hCG injection, and for a period of 14 days following hCG injection. The analysis of the samples using the developed hCG immuno-MS method showed that hCG could, in average, be detected for 7 days in serum following hCG injection. The window of detection in urine was 10 days following hCG injection. This was accomplished at LOQs as defined by World Anti-Doping Agency (WADA). Thereafter, hCG variant patterns a function of injected hCG drug was studied, followed by interpretation of hCG pattern complexity related to biological matrix.

Paper IV describes the comparison of the developed hCG immuno-MS method's performance to that of the existing reference immunometric method; the DELFIA assay. Windows of detection

and differences in hCG measurements were compared and discussed as a function of method selectivity and choice of matrix.

1. INTRODUCTION

1.1 Proteomics

The field of proteomics entails the study of all aspects of protein properties, from expression and profiling, to modifications and interactions. The information obtained from the identification and quantification of proteins can impact broadly on biology and medicine when used for clinical applications or biomedical research [1]. The enormous potential of protein biomarkers to revolutionize clinical practice and thus improve patient care through molecular based diagnostics has been thoroughly described [2,3]. Also the rapidly evolving peptide and protein drugs can be characterized and their pharmacokinetics effects studied based on applied proteomics [4,5].

Mass spectrometry (MS) has increasingly become the method of choice for analysis of complex protein samples, largely due to the development of soft ionization techniques such as electrospray ionization (ESI) and matrix-assisted laser desorption/ionization (MALDI) [1]. The suitability of MS analysis to complex proteomics studies is related to the unique features of the instruments in terms of resolution and mass accuracy, combined with sensitivity and the ability to generate specific mass spectra for the detected peptide ions [1]. In general two MS-based proteomics approaches applied for protein analysis are the top-down approach where intact proteins are analyzed, and the bottom-up approach which involves the enzymatic conversion of proteins into their constituent peptides [6]. Whereas the analysis of intact proteins is greatly challenged by the lack of sensitivity as a function of charge distribution and thus poor efficiency of ionization, the bottom-up approach has gained territory as substantially better sensitivity is provided by chromatographic separation of the generated peptides prior to MS analysis [7], hereby enabling efficient ionization of the separated peptides. This bottom-up strategy can further be divided into discovery or targeted experiments. Proteomics discovery experiments aim at identifying the detectable protein content of a sample and are referred to as “shotgun” proteomics. This is carried out through the interpretation of the generated mass spectra by database searching, and requires thus high performance of the analysis in order to provide proteomics data that can be fully interpreted to generate testable conclusions, regarding *i.e.* biomarker discovery, identification and characterization [1]. Figure 1.1 summarizes a general path for bottom-up proteomic analysis.

However, targeted proteomics is increasingly used in clinical validation and diagnostic method development, and for efficient and rational applicability in this setting some of the high

performance of the analysis is exchanged for higher throughput, robustness and simplicity [8]. For the targeted study of the behavior of known sets of proteins sensitive and accurate quantification can be obtained through the selected reaction monitoring (SRM) technique [9,10].

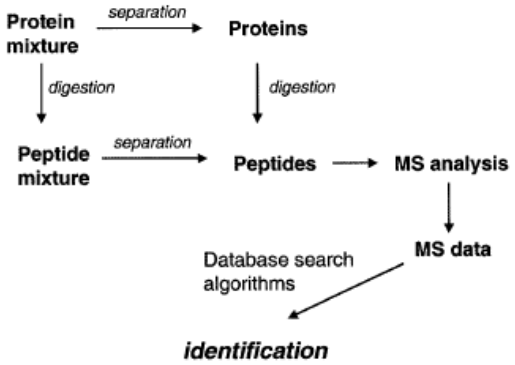


Figure 1.1 *General flow scheme for proteomic analysis (From reference [11], with permission)*

1.2 Targeted proteomics approach by SRM

1.2.1 Principle and background

In targeted proteomics by SRM the MS is programmed to detect a preselected protein or group of proteins. This technique is comparable to the selected ion monitoring (SIM) technique, in which the intensities of several preselected specific *m/z* values are recorded rather than the entire mass spectrum, resulting in increased sensitivity of the MS analysis. Further improvement of sensitivity is obtained through the extension of SIM detection into SRM detection.

The development of the SRM technique [12,13] came along with the development of the first triple quadrupole instruments in the late 1970s [14]. In clinical applications this has been a reference quantitative technique for the analysis of smaller molecules for 30 years [9]. However the recent years methodological developments have made targeted proteomics analysis by SRM-MS a method of increasing interest [15]. SRM assays are developed on triple quadrupole instruments (QqQ) to detect peptide ions diagnostic for the parent proteins, hereby enabling

sensitive, reproducible and quantitatively accurate measurements for the specific identification and quantification of target proteins [10,16,17]. The procedure entails the enzymatic conversion of proteins into peptides, followed by LC separation prior to SRM-MS analysis by the QqQ. Peptides entering the first quadrupole are mainly doubly or triply charged. In the collision cell they are further subjected to fragmentation, resulting in fragment ions. A few specific fragments are further monitored for detection and quantification purposes [9]. This will be more thoroughly explained in the following sections.

1.2.2 **Tryptic digestion and signature peptides**

The enzymatic conversion of proteins into peptides can be effectuated by trypsin, which is the most widely used approach [11]. The specific cleaving at the C-terminal of the amino acids lysine and arginine in the protein backbone generally results in peptide sequences of shorter size. In addition to the charge of the N-terminal of the peptides, the lysine and arginine residues impose a second charge on the peptides, making them suitable for electrospray ionization. These characteristics are favorable for MS detection of peptides.

Amongst the peptides generated from enzymatic cleaving of a protein, certain peptides are composed of unique amino acid sequences that are specific for the parent protein. These *signature peptides* serve as such as diagnostic representatives for the detection of the parent protein. Additionally, for accurate quantification of the target proteins, these signature peptides also have to be stoichiometric representatives. This requires that the proteolysis is complete, or at least has reached an end-point that is consistent among samples [18,19].

This detection of a unique structural component of the target molecules replaces the far more complicated detection of intact proteins, hereby enabling sensitivity and selectivity crucial for the quantification of low abundance proteins in complex biological matrixes. As such, this principle has been demonstrated by several research groups [16,20-23,18,19,24]. The advantages related to this strategy might lead to the increasing application of the SRM technology to larger molecules, such as proteins, for analyses wherein the specific identification and quantification of the target macromolecule is important.

1.2.3 **Analytical technique: LC-MS/MS**

The LC-SRM-based analysis of peptides is based on the complementary features and sequential organization of LC separation of peptides, electrospray conversion of charged peptides in the liquid phase to ions in the gas phase, and the selection and fragmentation of target peptides in the triple quadrupole mass spectrometer.

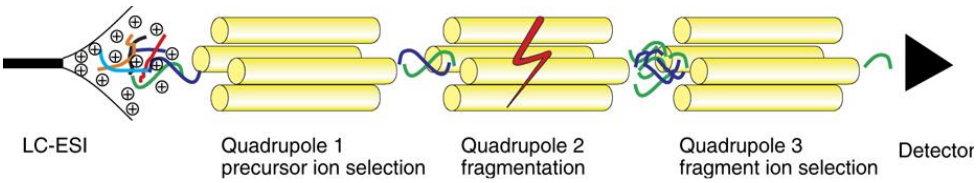
Of particular relevance in peptide analysis is the ability of the ESI, which is a soft ionization technique, to leave the peptides intact prior to entering the mass analyzer. Furthermore, the first quadrupole selects target peptide ions based on *m/z* values programmed to the QqQ (Figure 1.2 A), and introduces these to the second quadrupole where they are subjected to fragmentation by collision induced dissociation (CID). This fragmentation technique produces mainly cleavages along the protein backbone between the carbonyl oxygen and the amide nitrogen, resulting in b-fragment ions (counting from the C-terminal of the peptide) and complementary y-fragment ions (counting from the N-terminal of the peptide) (Figure 1.2 B). Depending on signal intensity and specificity of the generated fragment ions in the method optimization process, a selection of fragment ions to be monitored by the third quadrupole is made.

The discriminating character of the QqQ combined with the LC separation of peptides enhances selectivity and sensitivity of the LC-MS/MS method, which is fundamental in biomarker determination. An additional and highly important feature of the complementary features of LC-MS is the potential to multiplex the detection of a preselected group of proteins in one single run [25,26].

1.2.4 **Sample preparation: Immunoaffinity extraction proceeding MS analysis**

Although the discriminating ability of the MS to selectively lock on to preprogrammed *m/z* values and hereby exclude components of diverging *m/z* values is indeed effective, a sample preparation strategy is needed to enable accurate and sensitive analysis of low abundance target molecules in complex matrixes such as serum and urine. A traditional sample preparation technique much applied in proteomics is sample fractionation using either one- or two-dimensional gel electrophoresis, where proteins are separated according to molecular weight and/or on the basis of isoelectric point by isoelectric focusing (IEF) [11]. A protein depletion step can either be carried out independently or be combined with sample fraction depending on the abundance concentration of the target analyte. In protein depletion abundant serum proteins are

A



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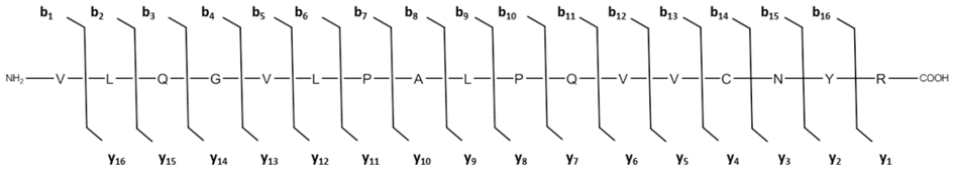


Figure 1.2 A) *SRM analysis on QqQ MS. Selection and filtering of co-eluting compounds according to m/z values, followed by fragmentation and m/z based selection of fragments for specific detection. (From reference [10], with permission)* ***B)*** *Representation of a peptide as a construct of amino acid building blocks. The specific cleavage of the protein backbone by CID generates complementary b- and y- ions*

removed using immunoaffinity depletion columns [27], hereby greatly reducing serum complexity. However, immunoaffinity enrichment of target analytes short-circuits the need for abundant protein depletion and fractionation before SRM-MS, and has emerged as a selective and compatible technique suitable for coupling to mass spectrometric detection allowing enrichment of a target protein as much as 1000-fold [28-30].

In a historical perspective the development of immunoaffinity-based MS approaches followed the demonstration of the first MALDI and ESI-MS protein analysis in the late 1980s [31-33] as a promising strategy for solving the obvious sensitivity issues related to the MS detection of macromolecules. Selective isolation and extraction of proteins is possible when selective and specific antibodies directed towards epitopes on the target protein exist. If the target consists of a

group of different proteins, this can be achieved by combining a selection of antibodies targeted towards the different proteins [34]. Another approach is the use one antibody that is targeted towards a common epitope on all the target proteins [35].

Different formats can be applied for immunoaffinity sample preparation preceding MS analysis, depending on whether the method aims at an on-line or off-line set-up. Immunoaffinity columns can be used for both set-ups. However, since tryptic digestion is a feature of the MS-based analysis of larger proteins, the on-line approach will be complicated. The off-line mode has been demonstrated for the purification of proteins prior to tryptic digestion and MS detection [36,37]. Other formats are the use of conventional 96-wells format plates, as applied for immunometric assays [35], and the immobilization of monoclonal antibodies to magnetizable particles (beads) for the extraction from larger samples volumes [38]. The enrichment of tryptic peptides by anti-peptide antibodies immobilized in nano-affinity columns has also been demonstrated [39]. This approach entitled SISCAPA (Stable Isotope Standards and Capture by Anti-Peptide Antibodies) is however restricted by the tryptic fragment selected for analysis, leaving no possibility to change or include other signature peptides to the method. Furthermore the antibodies targeted towards tryptic protein fragments in stead of proteins tend to be less antigenic based on size considerations.

Successful applications of quantitative peptide hormone analysis enabled by immunoaffinity extraction and MS detection (hereafter referred to as immuno-MS) have been demonstrated, such as the analysis of peptide hormones in doping analysis [34]. Other applications involving immunoextraction and MS detection of target proteins through diagnostic signature peptides have been shown for the analysis of protein biomarkers in clinical diagnostics [40-42,22].

The above argues the potential of the immuno-MS strategy as analytical methodology for the determination of target proteins in complex biological matrixes.

1.2.5 **Quantification of proteins**

Due to differences in ionization efficiency between compounds it is not possible to give accurate quantitative measurements of target compounds based on their MS signal intensities alone. As such MS is not inherently a quantitative technique, and to achieve accurate and precise

quantitative measurements in targeted proteomics the established principle of stable-isotope dilution (SID)-MS can be employed [43,44].

SID-MS entails the addition of a known amount of isotopically labeled standard to the sample, having the same physicochemical proprieties as the analyte. Chromatographic co-elution and equal efficiency of ionization is thus obtained for the internal standard (IS) and the target analyte. They are however separated in the mass spectrometer due to differences in mass (Figure 1.3).

The appropriate point in the workflow to add an IS to the sample is dependent on the nature of the standard used (Figure 1.3). *Proteins standards for absolute quantification* (PSAQ) are recombinant isotope-labeled protein analogues to the target proteins [45], and should thus be added immediately (or as soon as possible), in defined amounts, to the sample. This is also the case for the FLEXIQuant method, where labeled protein analogues are flanked by a FLEX peptide which is used for their calibration [46]. Both these strategies will completely overcome any problems associated with differential digestion, which is a major issue for many quantification strategies. However, they require quantification of each standard separately, which limits their strength as multiplexed strategy, increasing costs and decreasing throughput [43].

Multiplexed quantification using artificial QCAT (concatenation of tryptic peptides) proteins (QconCAT) [47] and the *Protein Epitope Signature Tag* (PrEST) [48] quantification strategy are techniques designed for multiplexed quantification. Their construction requires addition of standard prior to digestion, in order to release the signature peptide to the sample (Figure 1.3). Although both standards contain the target signature peptide in their protein backbone, they are not protein analogues to the target proteins; the QconCAT protein is designed and synthetized to contain the desired signature peptides in one larger protein [47], whereas the PrEST peptides are shorter protein fragments produced by the Human Protein Atlas (http://www.proteinatlas.org/) where they are used as antigens for antibody production [48]. Their relatively simple structure (linear protein/peptide sequences) ensures near complete digestion. This, however, might not be the case for the target proteins. Together with the strategy of the *absolute quantification peptides* (AQUA), these strategies thus require strict control of completion of digestion in order to provide accurate quantitative ratios for the added amount of IS and the generated signature peptide being a stoichiometric representative of the target protein.

The AQUA peptides are isotopologes to the target signature peptides [49,50], and are thus primarily added before LC-MS analysis. Given that the process of digestion is thoroughly explored and validated, and is further proven to reach an end-point, the use AQUA peptides can provide advantages such as limited in-house expertise in the preparation as these can be purchased from several commercial companies. All in all the simplicity of use combined with reliable quality, reasonable costs and accessibility are advantages that should be taken into account when choosing internal standards for assays designed with inter-laboratory utility in mind.

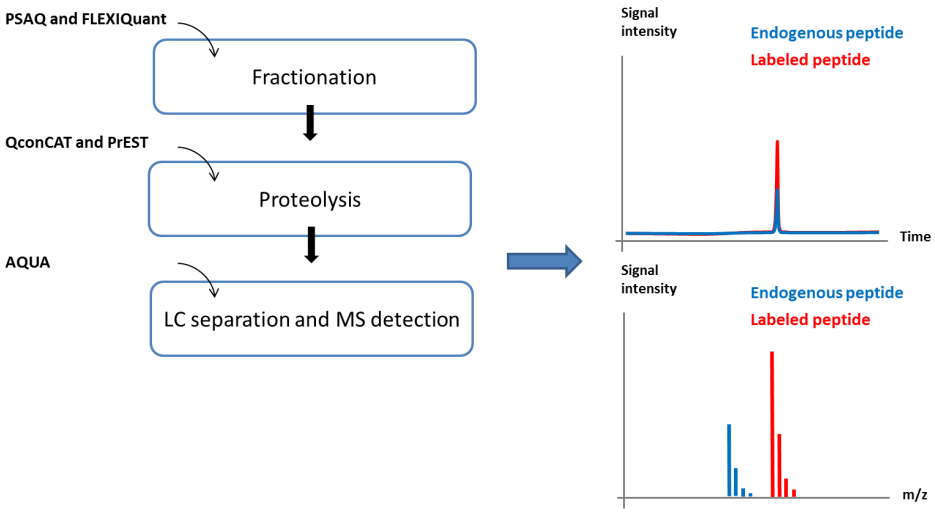


Figure 1.3 *Strategies for absolute quantification of target proteins by various SID techniques. The addition of the five different types of standards to the sample is dependent on the nature of the standard; if the signature peptide needs to be released from the standard prior to LC-MS/MS analysis, then the standard is added prior to enzymatic digestion (PSAQ, FLEXIQuant, QconCAT and PrEST). Only standards that are protein analogues to the target proteins can be added prior to prefractionation of the proteins (PSAQ and FLEXIQuant).The synthetic isotopically labeled signature peptides (AQUA peptides) are added prior to LC-MS/MS analysis*

1.2.6 **Immuno-MS strategy**

On the basis of what has been presented above, a strategy for absolute quantification of multiple target proteins based on immunoaffinity extraction and bottom-up MS detection can be outlines as summarized in Figure 1.4. This strategy involves SID-MS using AQUA peptides for quantification purposes, prior to LC separation and tailored SRM-MS detection of signature peptides.

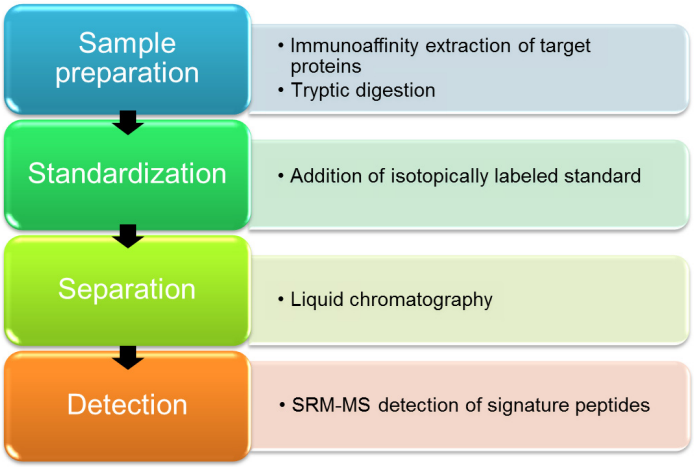


Figure 1.4 *Immuno-MS strategy for quantification of low-abundance protein biomarkers in complex biological matrixes*

1.3 **Human chorionic gonadotropin: a diverse biomarker**

Effective management of patients is funded on the ability to provide early and reliable diagnosis and prognosis. Accurate determination of established and reliable biomarkers aims at providing clinical information valuable for the interpretation of disorders. Further monitoring of disease progression and response to therapy can often be measured in terms of biomarker presence, up- or down-regulation. Since many biomarkers are proteins the use of targeted proteomics applied in

a clinical setting is promising, for all the reasons stressed in previous sections. However, the application of MS-based clinical diagnostics on macromolecules has only been tailored for a few proteins. The human chorionic gonadotropin family represents a diverse group of proteins whose diagnostic value has been well documented. As such this is an interesting candidate for tailored MS-based detection using the targeted proteomics approach.

1.3.1 **Molecular structure and biochemistry**

hCG is a highly glycosylated protein (37.5 kDa), and is part of the glycoprotein hormone family which also includes luteinizing hormone (LH), follicle-stimulating hormone (FSH), and thyroid-stimulating hormone (TSH). These hormones are heterodimers comprising a common α-subunit (92 amino acids) and a specific β-subunit. The latter confers biological activity, and is specific for each hormone. The hCG β-subunit is 145 amino acids long. The assembly (Figure 1.5) of the two hCG subunits is non-covalent, and dissociation of the subunits might thus occur. Internal stabilization is conferred by three disulfide bonds forming a cysteine knot. One-third of the molecular mass is made up by eight carbohydrate moieties, of which six are attached to the β-subunit and two to the α-subunit (Figure 1.7). These sugar moieties are either N-linked (linked to asparagine residues) or O-linked (linked to serine residues), and vary in size, possibly resulting in hyperglycosylated variants. Within each subunit, several disulfide bonds contribute to the tertiary molecular structure. This results in the formation of several loops in the protein backbone, which have been given designations according to their position counting from the N-terminal (Figure 1.5). The c-terminal peptide (CTP) constitutes the part from amino acids 114-145 in the hCG β-subunit backbone, and this is a highly glycosylated region in the protein with four O-linked carbohydrate groups [51,52].

The hCG molecule is not a single molecule although it is often referred to as one. It is a heterogeneous molecule that can be diverted into variants having different cell origins and thus different biological effects [53]. They all share the same protein backbone core, referred to as hCG β-core fragment (Figure 1.7), but dissociation of heterodimers into free subunits, differences in glycosylation, and nicking in the protein backbone might occur, resulting in a variety of hCG molecules. These variants are schematically presented in figure 1.7.

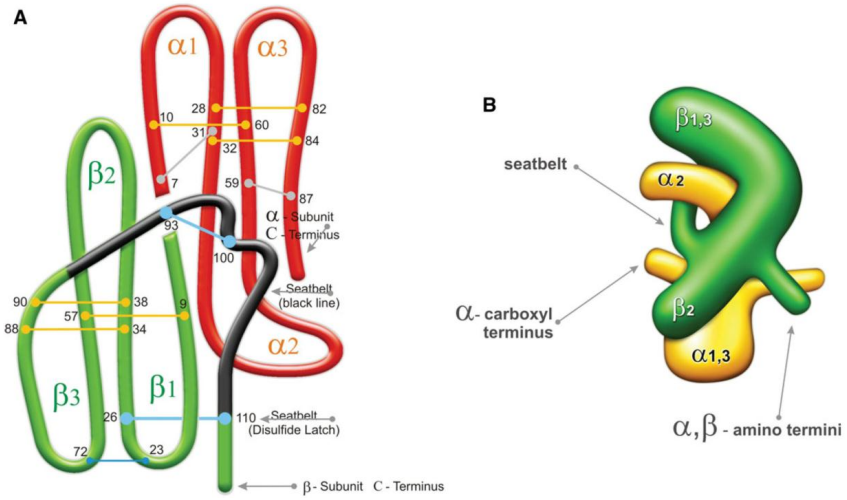


Figure 1.5 *A) Assembly of α- and β-subunit to form hCG heterodimer B) Spatial representation of the subunit assembly in which the hCG α carboxy-terminal extension penetrates the hCG β and is locked in a seatbelt configuration by the β-subunit. (From reference [54], with permission)*

The main hCG variant is the intact hCG (αβ heterodimer), which also displays the main biological function provided by hCG. Degradation of this molecule by metabolism is mainly the cause of variation in hCG structure. Once released from the cell to the circulation (Figure 1.6) degradation processes occur, resulting in degraded hCG variants that are rapidly cleared from the circulation [52,55]. Whereas the intact hCG has a metabolic clearance half-life of ~ 36 hours, the corresponding half-life of the free hCG β-subunit is ~ 4 hours. The degradation variants are thus more likely to be found in urine than in serum [55].

When cleaving of the bond linking two amino acids in the protein backbone occurs as part of the degradation process, this is called nicking. This occurs mainly in the amino acids 44-48 in the backbone, and the most common variants are the ones displaying nick between amino acids 47/48 and 44/45 [35]. Nicking of hCG happens in both normal and pathological conditions, and seems to be executed by proteases present in the circulation and the kidneys. The precise types of

proteases, however, remain yet to be elucidated [56-58]. Further degradation of the molecule by proteases results in the ultimate hCG degradation variant, the hCG β-core fragment.

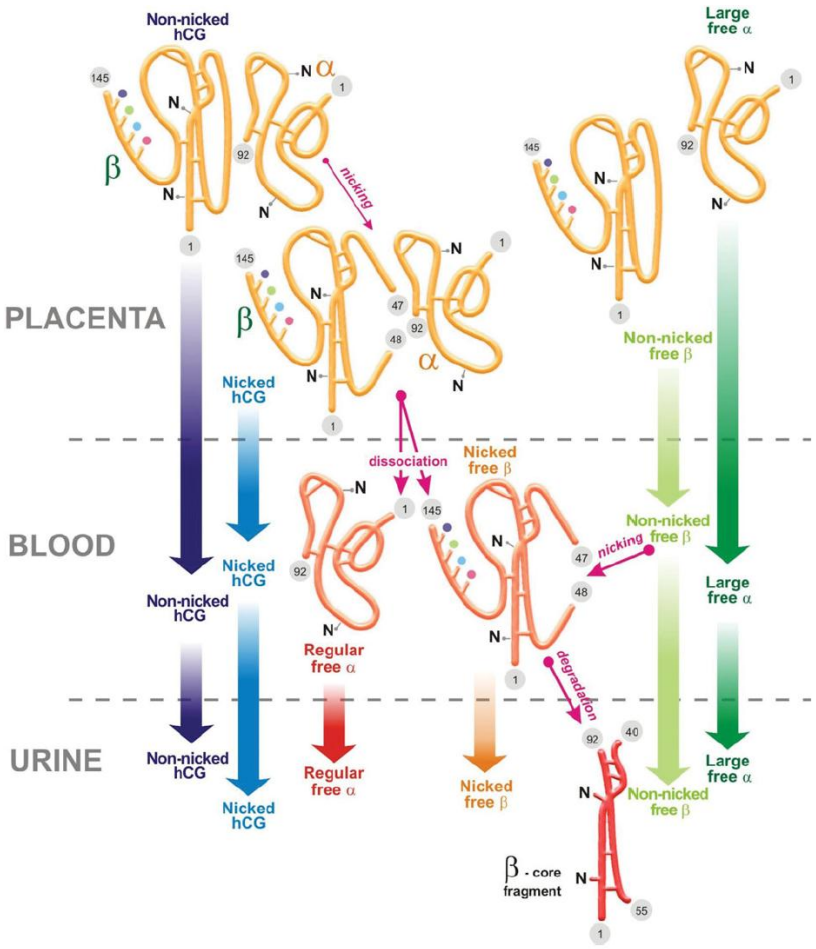


Figure 1.6 *hCG variants in placenta, blood and urine. Degradation pathways, dissociation and nicking of the hCG molecules. Large free α refers to hyperglycosylated α-subunit. (From reference [54], with permission)*

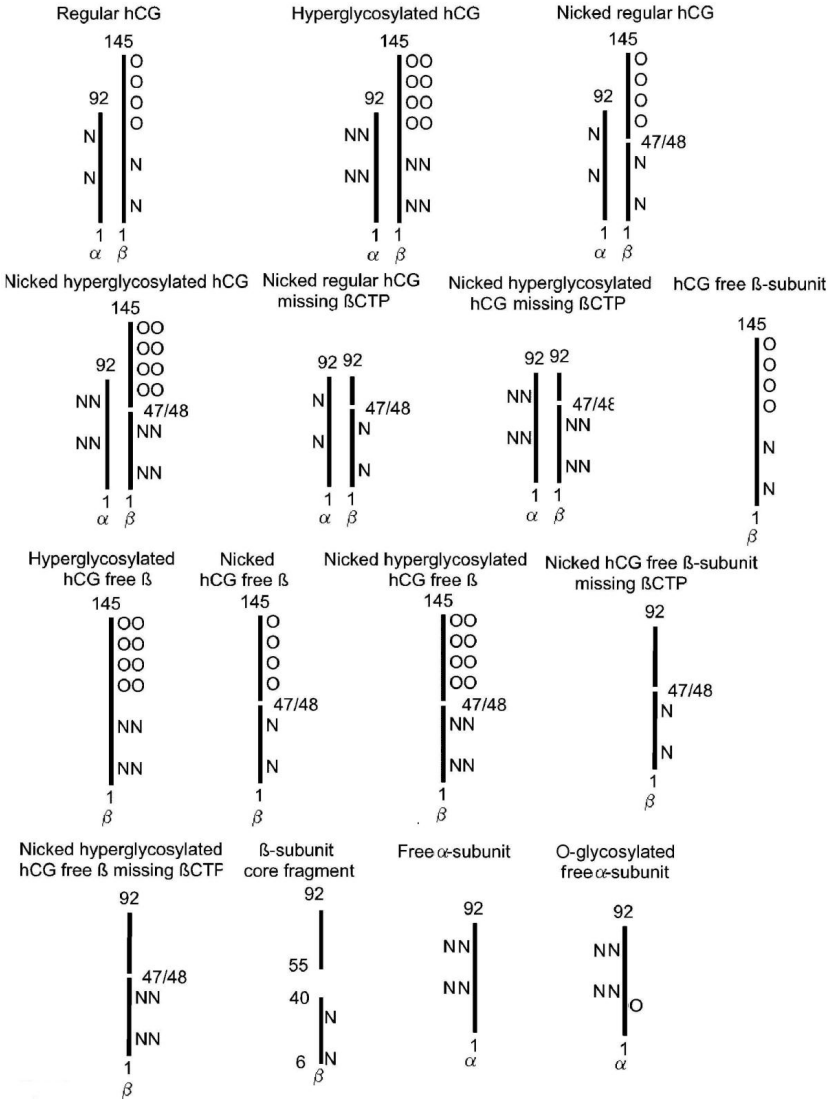


Figure 1.7 *Outline of the structure of the 15 common hCG variants present in serum and urine samples in either pregnancy, gestational disease or other malignancy. Numbers refer amino acid numbers, O refers to O-linked and N to N-linked oligosaccharides. OO and NN refer to large or hyperglycosylated oligosaccharides. βCTP is the C-terminal segment (residues 93-145) on the hCGβ-subunit (From reference [55], with permission)*

The different hCG variants presented in Figure 1.7 include all possible variants as a function of dissociation, nicking, degradation and hyperglycosylation. This extensive variation is not particularly relevant per se. Furthermore, from a methodological point of view the differentiation based on variation of the sugar groups is quite complicated to execute. The structural variation is thus often limited to that of the differences in the protein backbone, resulting in the pragmatic classification of hCG molecules into the intact hCG, the free hCG β-subunit, the hCG β-core fragment, and the nicked variants [59,60]. This classification has been recognized, and standards have been produced for these hCG variants including the α-subunit [59].

The area of a molecule that is specifically recognized by antibodies is referred to as the epitope of the molecule, and several epitopes might exist on the same compound. For hCG, the different epitopes are classified according to the epitope cluster that they are in the proximity of, primarily dividing the epitopes into groups of α-epitopes, β-epitopes or c-epitopes (Figure 1.8) [35]. Within these α-, β- and c-epitope groups further sub-classification more precisely defines the epitopes' exact position. The molecular epitope structure of hCG has been a topic of interest for some years [35] since this family of diverse molecules poses challenges in terms of antibody-based detection. This is related to the large number of hCG epitopes as a function of structure and subunit assembly diversity. This will be more thoroughly discussed below.

1.3.2 **Clinical proprieties**

The function of hCG is mainly to maintain the progesterone production of corpus luteum during early pregnancy. As such hCG is principally produced during the full course of pregnancy, hereby controlling placenta, uterine and fetal growth and/or differentiation [53]. It is thus the hormone that is used for pregnancy detection and monitoring of pregnancy. Quantitative determinations of hCG are also used for assessment of disorders of pregnancy, hereunder the prediction of complications especially in early pregnancy, e.g. pregnancy loss and ectopic pregnancy [52].

hCG is also an extremely sensitive and specific marker for gestational throphoblastic disease (GTD, pregnancy-related tumors) and for some germ cell tumors of the testis. The sensitivity and specificity of hCG measurements in GTD approach 100%, while approximately 50% of patients with testicular germ cell tumors have elevated hCG levels. Furthermore, for patients with various non-trophoblastic neoplasms, elevation of free hCG β-subunit occurs in 30-70% [61].

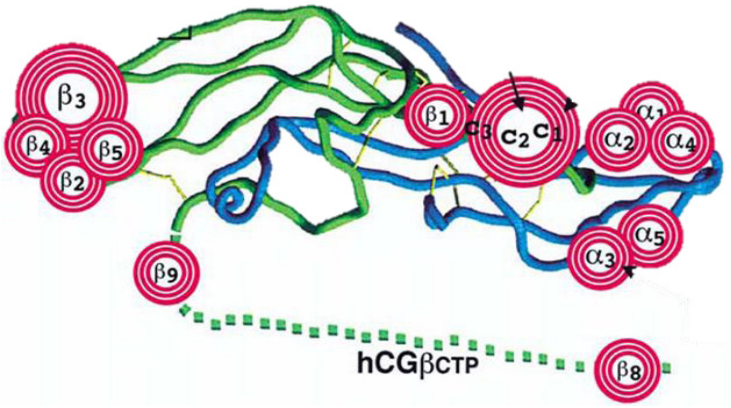


Figure 1.8 *3D Epitope map of hCG. The cluster of the α-epitopes, β-epitopes and c-epitopes are emphasized in red. Since this is a 3D epitope map, not all the specific epitopes are visualized in the figure. In the 3D dimension some of the epitopes are located behind the others. (From reference [35], with permission)*

The function of hCG in cancer development is not fully elucidated, however the role as cancer promoter has been established [53] and the ability to promote growth of tumor cells by preventing apoptosis has been suggested [62].

Together with Down syndrome screening [63] the above constitutes the major clinical use of hCG as a biomarker. However other conditions might also produce variable levels of hCG. Extremely low levels of hCG have been detected as a consequence of hCG production by the pituitary gonadotrope cells during the normal menstrual cycle [53]. Additionally pituitary hCG can be produced at detectable levels during menopause [53]. Recently there has been reports on "familial hCG syndrome" in which case only biologically inactive variants were detected [64]. These hCG producing conditions have no pathological consequences, but are nevertheless causes of concern as they produce positive responses for hCG testing in men and non-pregnant women, leading to confusion and in worst case inappropriate treatment of healthy patients [65].

In addition to the biomarker proprieties of endogenously produced hCG, the pharmacological effects of hCG injections are mostly exploited to induce ovulation in infertility treatment [52]. For men, a few more rare indications exist where to ability to stimulate testosterone production has been used for selected cases of hypogonadotropic hypogonadism, prepubertal cryptorchidism not due to anatomical obstruction, and in combination with other drugs for male infertility treatment [66].

1.3.3 **hCG in doping analysis**

The most "famous" benefit of hCG injections is the stimulation of testosterone production in males. The anabolic effect of the increased endogenous androgen production generates physical advantages in male athletes, particularly in power sports, and is as such a means of indirect androgen doping [67,68]. Additionally this effect can be exploited to increase endogenous testosterone production that has been suppressed during and after prolonged use of anabolic steroids [67-70]. hCG is thus included in the World Anti-Doping Agency (WADA) list of prohibited substances, both for in- and out-of-competition testing [71].

The natural presence of this hormone during pregnancy combined with the lack of proven beneficial effects in females make the use of hCG illegal only for male athletes [67-70]. WADA states that the finding of hCG in the urine of male athletes at concentrations higher than 5 IU/L may be an indicator of hCG use for doping purposes, and should thus be reported as adverse findings [72]. Due to the complexity of hCG isoform composition in urine and the reported association of some hCG molecular forms with pathophysiological conditions such as cancer, consideration must be given to plausible causes, other than doping, that can produce elevated hCG concentrations in urine samples from male athletes [72].

The WADA guidelines for reporting and management of hCG finding indicate that for the initial testing procedure (screening) laboratories should apply immunoassays capable of detecting the total hCG content in urine, which should include many of the molecular forms of hCG found in urine (e.g. intact hCG, free hCG β-subunit, nicked hCG and hCG β-core fragment). The confirmation procedure should in contrast apply immunoassays that specifically detect the intact hCG exclusively [72].

1.3.4 **hCG detection and immunoassays**

For both cancer diagnostics and doping analysis the hCG detection is currently based on immunometric methods [73], such as the conventional sandwich principle based immunoassays [74-77]. The sandwich principle entails the combination of a capture and a tracer antibody forming a complex with the target analyte. The tracer antibody is often linked to an enzyme; when the enzyme's substrate is added to the antibody-antigen complex the subsequent reaction produces a detectable signal, most commonly a color change in the substrate; hence enzyme-linked immunosorbent assays (ELISA). This signal is thus proportional to the amount of target analyte in the samples. If the assays are targeted towards several analytes the generated response will represent the sum of all detected analytes. The uniform character of the response prevents the differentiation between the various molecules that are captured and thus detected, including any unspecific binding of interfering proteins.

Depending of the hCG fingerprint expressed during various clinical conditions, different immunoassays are selected for adequate hCG detection. For regular pregnancy detection, simple over-the-counter (OTC) assays are well suited since the production of hCG during pregnancy development is quite high [78]. In cancer diagnostics, tailored selectivity is often required in order to discriminate certain cancer conditions from others [75-77,61]. The selectivity of immunoassays is dependent of the selectivity characteristics of the antibodies used; the capture and the tracer antibody have to recognize different non-overlapping epitopes on the target molecule. In this context, the family of hCG molecules has been shown to be greatly challenging due to the large variability in structure of these molecules. This has lead to reporting of substantial inter-assay variability [79,80,75,81-83], non-standardized hCG measurements [84,85,83] and known cases of false positive and false negative hCG measurements [86-89] leading to inappropriate patient management [86,65]. Additionally the phenomenon of “phantom” hCG [87] and cases of interfering heterophilic antibodies [90,86] have been described, further adding insecurity to the hCG measurements effectuated by immunoassays. The recognition of these problems related to hCG measurements has resulted in mobilization of efforts in order to standardize hCG measurements through generating a clear nomenclature [35] and providing adequate hCG standards for the different variants [59,60,91]. Furthermore, the epitope mapping of the hCG molecules [35] in combination with the characterization of the selectivity of various anti-hCG antibodies towards the different hCG molecules [35] is currently in process. These

combined efforts are hoped to generate answers allowing standardization and tailoring of immunoassays for proper hCG measurements.

Although immunometric assays are currently used for hCG detection in doping analysis, it is indicated by WADA's International Standard for Laboratories that MS should be the analytical technique of choice for confirmation of prohibited substances. Furthermore the limitations of the application to anti-doping testing of the currently commercially available hCG immunoassays, mainly developed for pregnancy testing and cancer biomarker detection, are also recognized in the doping testing arena. A method that can be implemented in a harmonized way and which allow not only the quantification but also the specific identification of the target analyte brings obvious benefits.

1.3.5 **hCG and mass spectrometry**

The work of characterizing the hCG β-subunit by both MALDI-MS [92,93] and LC-MS/MS [94,95] have been previously described by others. This was succeeded by the LC-MS (/MS) analysis of the carbohydrate groups of the hCG molecule [96,97]. Thereafter the principle of immunoaffinity extraction of hCG prior to LC-MS/MS analysis for use in doping analysis was demonstrated by Gam *et al.* [36,37]. They described the development of a method based on immunoextraction using an immunoaffinity column in the off-line mode, followed by LC-MS detection of the intact hCG, intended for use as confirmatory hCG test in doping analysis [36]. The specificity of the method was limited to the identification of the intact hCG molecule and the method was not validated for quantification measurements. Additionally, the experimental set-up employing an immunoaffinity column in the off-line mode was complicated and time-consuming, leaving inter-laboratory implementation of the method practically impossible.

All in all the above constitutes a solid foundation for the development of a MS-based method for determination of various hCG variants in complex biological matrixes.

2. AIM OF THE STUDY

The clinical impact of the hCG molecules and corresponding necessity of accurate hCG measurements, combined with the problems associated with immunometric hCG detection, make hCG an interesting candidate for tailored and differentiating MS detection. The intention of the present study was thus to develop a highly specific MS based method for determination of hCG and related molecules using the targeted proteomics approach. In order to achieve this, the following challenges had to be addressed:

- Establishment of adequate signature peptides (**Paper I**)
- Reduction of proteome complexity dominating the biological matrixes in order to grant access for the MS to the low abundance target proteins (**Paper I**)
- Design of selective and specific MS detection (**Paper II**)
- Implementation of adequate quantification strategy and validation of developed method (**Paper II**)
- Demonstration of method applicability in clinical relevant scenarios
    - Clinical diagnostics (**Paper II**)
    - Evaluation of anti-hCG antibodies selectivity and specificity for tailored assays (**Paper V**)
    - Doping analysis (**Paper III**)
- Comparison of the developed methods performance to existing reference-quality method (**Paper IV**)

3 RESULTS AND DISCUSSION

3.1 Identification and qualitative differentiation between hCG variants using LC-MS

The targeted proteomics approach is based on the detection of unique signature peptides that are stoichiometric representatives of their respective parent proteins. When the target proteins are subjected to tryptic digestion, a vast number of peptides are produced. For proper selection of adequate signature peptides from this complex peptide mixture, theoretical data base selection of candidate peptides precedes the experimental peptide selection (**Paper I**).

3.1.1 Theoretical selection of signature peptides

For the hCG molecules the theoretical selection of signature peptides entailed two important considerations; first, the careful selection of signature peptides that enabled differentiation between the hCG molecules and other structurally similar proteins. Second, the differentiation between one hCG variant from another. This could only be possible if the structural differences in the respective hCG molecules were in fact represented in the signature peptides.

The signature peptide preselection was carried out in three steps: **1)** In-silico tryptic digestion of the hCG β-subunit was performed using the ProteinProspector (http://prospector.ucsf.edu), a tool for MS/MS based proteomics. This produced a list of 15 peptides, from which only the peptides without missed cleavage sites were selected (Table 3.1). Peptides displaying one or more missed cleavage sites are harder to produce reproducibly, and were thus considered unsuitable for quantitative measurements.

2) For the evaluation of peptide specificity a sequence query in the National Center for Biotechnology Information (NCBI) was performed by the search engine Basic Local Alignment Search Tool (BLAST). This search excluded any peptide containing amino acid sequences that could be found in other human proteins, resulting in a substantially shorter list of specific peptides that were all unique and diagnostic of the hCG β-subunit.

3) A final theoretical elimination step was carried out, based upon the knowledge of which peptides contained N- and O-linked sugar moieties. These peptides containing carbohydrate attachments of varying size were likely to cause problems for both the separation and detection of the peptides. First, the hydrophilic properties of the glycopeptides affect the retention on the separation column, hereby increasing the risk of signal suppression and interfering noise due to

co-elution with the injection front. Additionally, the selective character of the MS would exclude any peptide having *m/z* values different to those preprogrammed to the instrument, as would be the case for the peptides with varying carbohydrate composition. This consideration excluded five peptides of specific protein backbones that all contained at least one carbohydrate group. All in all, these elimination processes resulted in a list of candidate signature peptides (Table 3.1) that would be suitable for detection of the hCG β-subunit, from a theoretical point of view.

Table 3.1 *List of peptides generated from in-silico digest of the specific hCG β-subunit. All peptides are fully digested (contains no missed cleavage sites) and are designated by peptide number counted from the N-terminus of the protein backbone. The evaluation of each peptide as candidate signature peptide is specified in the column to the right.*

Peptide	Amino acid position	Amino acid sequence	Selection
T1	1-2	SK	Unspecific
T2	3-8	EPLRPR	Unspecific
T3	9-20	CRPINATLAVEK	Specific, but N-linked carbohydrate
T4	21-43	EGCPVCITVNTTICAGYCPTMTR	Specific, but N-linked carbohydrate
T5	44-60	**VLQGVLPALPQVVCNYR**	Signature peptide candidate
T6	61-63	DVR	Unspecific
T7	64-68	FESIR	Unspecific
T8	69-74	LPGCPR	Unspecific
T9	75-94	**GVNPVVSYAVALSCQCALCR**	Signature peptide candidate
T10	95	R	Unspecific
T11	96-104	**STTDCGGPK**	Signature peptide candidate
T12	105-114	**DHPLTCDDPR**	Signature peptide candidate
T13	115-122	FQDSSSSK	Specific, but O-linked carbohydrate
T14	123-133	APPPSLPSPSR	Specific, but O-linked carbohydrate
T15	134-145	LPGPSDTPILPQ	Specific, but O-linked carbohydrate

However, as the family of hCG molecules comprises known variations in the structure of the specific β-subunit, signature peptides representing this intra-variability had to be deduced. This was addressed through a study of the structure in the protein backbone where the structural differences appeared. Since the hCG α-subunit in unspecific (can be found in other hormones as well), and since the intact hCG (α and β heterodimer) and the free hCG β-subunit share the same unmodified hCG β-subunit, these two variants cannot be differentiated. They will thus be detected through the same signature peptide. Similarly, the nicked hCG heterodimers will not be distinguished from the free nicked hCG β-subunits. The nicked variants, however, might display nicking in the protein backbone at two known sites: between amino acid number 44 and 45, and between amino acid number 47 and 48. Each nicked molecule normally appears with one nick. These nicking sites had to be expressed in the signature peptides, as to properly detect all nicked variants, and to distinguish between non-nicked and nicked hCG. As for the smallest of the hCG molecules, the hCG β-core fragment consists of two amino acid chains (amino acid position 6-40 and 55-92), covalently connected by four disulfide bonds. This hCG variant should therefore have a signature peptide that is cleaved from the end of one of these sequences. The final candidate signature peptides that theoretically allowed differentiation between the described hCG molecules are listed in Table 3.2.

The challenge of differentiating structurally similar proteins is visualized in this short list of candidate signature peptides; apart from the hCG β-core fragment that is represented by two potential signature peptides, only one signature peptide will fully distinguish the hCG β-subunit and the two nicked variants. This is due to the single nick (at two potential locations) in the protein backbone that constitutes the only structural difference between these proteins; their total protein masses and total amino acid sequences/compositions are identical. As the difference in protein structure increases amongst a group of proteins, so will the number of candidate signature peptides.

Table 3.2 *List of candidate signature peptides for the described hCG variants. Each signature peptide has been designated according to protein origin, location in the tryptic sequence generated when counting from the N-terminus, and protein modification. The respective parent proteins are listed in the column to the right.*

Signature peptide	Amino acid position	Amino acid sequence	Parent protein
βT5	44-60	VLQGVLPALPQVVCNYR	Intact hCG
			Free hCG β-subunit
nβT5 44/45	45-60	LQGVLPALPQVVCNYR	Nicked hCG 44/45
			Free nicked hCG 44/45
nβT5 47/48	48-60	VLPALPQVVCNYR	Nicked hCG 47/48
			Free nicked hCG 47/48
cfβT5	55-60	VVCNYR	hCG β-core fragment
cfβT9	75-92	GVNPVVSYAVALSCQCAL	hCG β-core fragment
αT2	36-42	AYPTPLR	hCG α-subunit

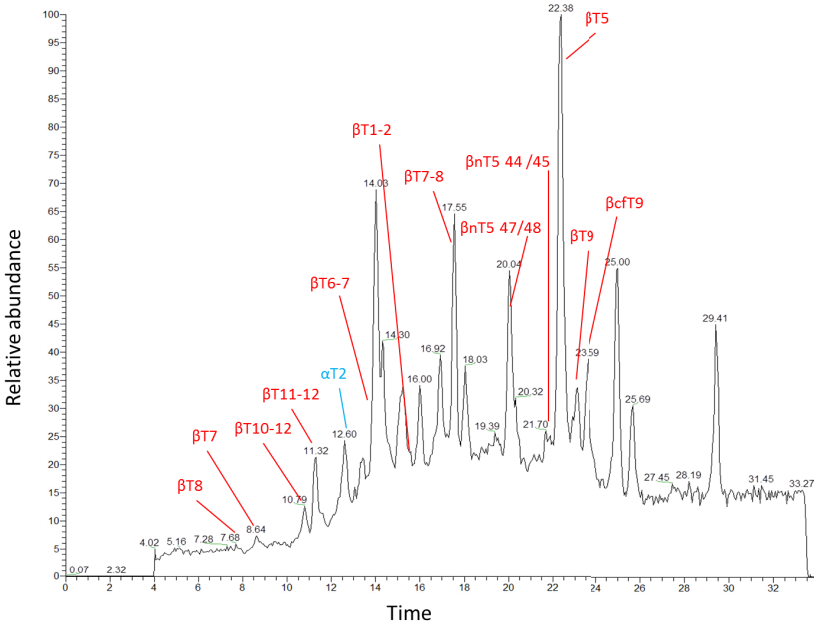
When it comes to the differentiation between heterodimers and their dissociated free subunits, as for the intact hCG and its free hCG β-subunit, this cannot be performed directly with the chosen strategy. However, there are indirect approaches within this strategy that can be explored, such as the establishment of the ratio of the detected β- and α-subunit of a heterodimer. If, for a certain sample, the detected β- and α-subunit signal produces a ratio that exceeds this known heterodimer ratio, then the excess signal of detected β-subunit can be contributed to free β-subunit. To explore this approach, a signature peptide of the hCG α-subunit had to be established (listed at the bottom of Table 3.2). It must be emphasized that since the α-subunit of the hCG molecules is the same as that for the luteinizing hormone, follicle-stimulating hormone and thyroid-stimulating hormone, there is always a possibility that parts of the detected αT2-signal might result from these structurally similar molecules, unless this has been prevented one way or another. This approach is therefore best suited for heterodimers of unique subunits. This will be further explored later in the thesis.

3.1.2 **LC-MS analysis: hCG peptide mapping and detection of signature peptides**

A solution containing most hCG variants (intact hCG, free hCG β-subunit, nicked hCG, and hCG β-core fragment) was subjected to tryptic digestion in order to produce peptides that were subsequently analyzed in a gradient run on the LC-SQ system using 20 mM formic acid and MeCN on a BioBasic C8 (50 x 1 mm) column. Since the hCG molecules contain several disulfide bonds, reduction and alkylation of the cysteine residues had to be performed prior to tryptic digestion, resulting in the addition of a carboxy-methyl group to each cysteine residue.

Peptide mapping of the analyzed hCG protein digest mixture was performed essentially by matching observed peptide masses detected through a broad scan performed by the SQ (*m/z* interval 350-1250) to the theoretical *in-silico m/z* values generated by ProteinProspector (Figure 3.1). This *in-silico* search was set to include 0, 1 and 2 missed cleavage peptides, in order to enable identification of as many eluting peaks as possible. This preliminary peptide mapping was succeeded by MS/MS experiments using an ion trap in order to verify the assumed identities of the hCG peptides. Observed b- and y-fragment ions were matched against theoretical fragment ions generated by ProteinProspector (Figure 3.2). A total of 13 peptides (containing 0-2 missed cleavages) were identified, covering most of the amino acid sequence 1-114 of the hCG β-subunit (Figure 3.1). This included the T5 signature peptide which is shown in Figure 3.2 accompanied by its recorded MS/MS spectrum. Additionally the two nicked signature peptides nT44/45 and nT47/48, the cfT9 signature peptide of the hCG β-core fragment, and the αT2 peptide of the hCG α-subunit were identified. The ionextraction chromatogram of the identified signature peptides derived from the broad scan is visualized at the bottom of Figure 3.1.

When it comes to the C-terminal of the hCG β-subunit (amino acids 115-145), this part will theoretically generate three tryptically derived peptides (T13, T14 and T15), which are highly glycosylated as they contain serine residues with O-linked sugar moieties attached. The theoretic tryptic peptides T3 and T4 are also glycosylated, but contain N-linked sugar groups. The size of these carbohydrate groups is not constant, and the ProteinProspector thus generates *m/z* values based on the protein backbone, i.e. disregarding the glyco-masses. These peptides were thus of unknown *m/z* values, and as such too complicated to identify. However, since all the theoretically selected signature peptides had been identified, no further effort was made to complete the identification of the peptides that had not been identified.


Relative abundance
Time
βT5
βT1-2
βnT5 44 /45
βT7-8
βcfT9
βnT5 47/48
βT9
βT6-7
αT2
βT11-12
βT10-12
βT7
βT8
22.38
14.03
17.55
20.04
25.00
29.41
14.30
16.92
18.03
23.59
16.00
20.32
25.69
19.39
21.70
12.60
11.32
10.79
8.64
4.02
5.16
7.28
7.68
0.07
2.32
27.45
28.19
31.45
33.27

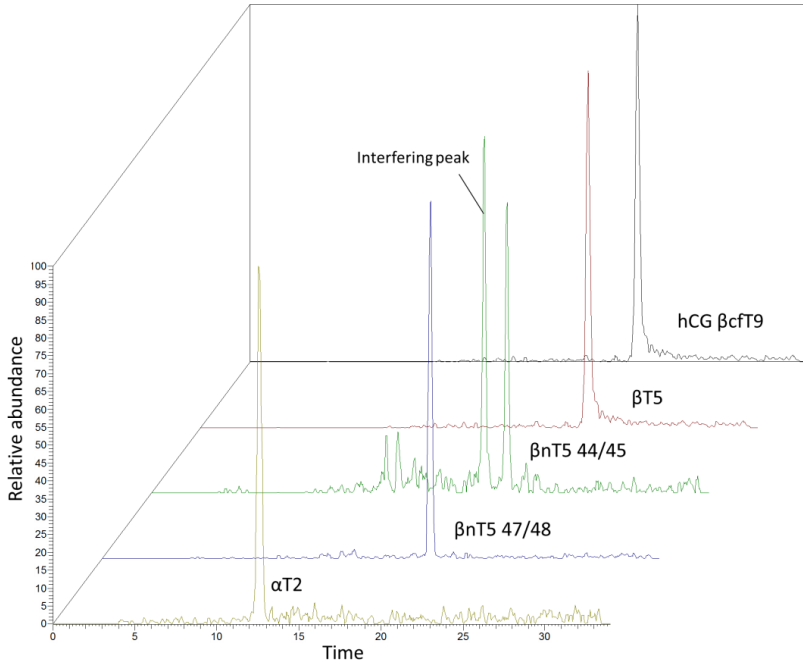

Relative abundance
Time
Interfering peak
hCG βcfT9
βT5
βnT5 44/45
βnT5 47/48
αT2

Figure 3.1 *(On page 36) Top: Peptide mapping of hCG. Chromatogram of a broad scan (m/z 350-1250) of the tryptic peptides derived from proteolysis of a mixture of hCG. All peptides of the hCG β-subunit that did not contain sugar groups attached to the protein backbone were identified. Only one peptide of the hCG α-subunit was identified. Bottom: Ion extraction chromatogram derived from the broad scan presented in the chromatogram above. of the selected signature peptides of the hCG β-subunit, the hCG α-subunit, the hCG β-core fragment, and the two nicked hCG variants*

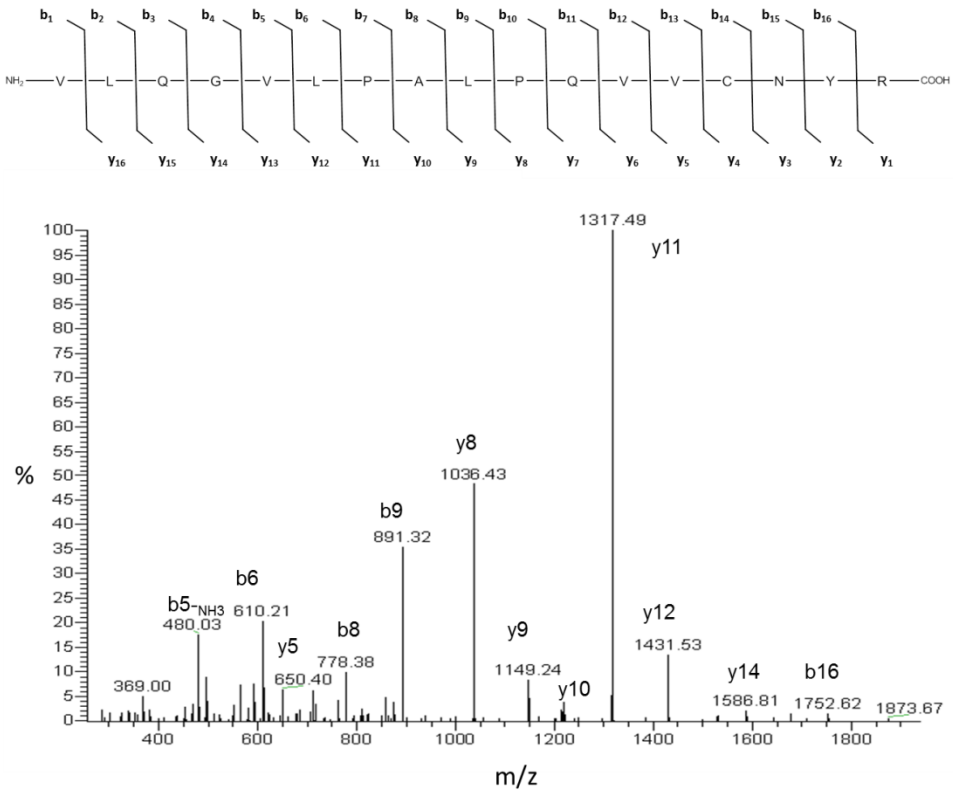


Figure 3.2 *MS/MS spectrum obtained from the fragmentation of the hCG β-subunit signature peptide, βT5. The most abundant b- and y-fragment ions are annotated. The amino acid sequence with the corresponding b- and y-fragment ions is shown at the top of the figure.*

Based on these experiments, an MS detection program in the selected ion monitoring (SIM) mode was set up. The SIM detection of each peptide was made on the basis of either doubly or triply charged peptides, depending on what charge state generated the highest signal response in the SQ. This varied amongst the different peptides analyzed by the same SQ mass spectrometer, but also varied for each individual peptide when transferring the LC separation to precede the ion trap detection for the MS/MS experiments.

3.1.3 **Pregnyl as hCG source**

The available hCG source was the pharmaceutical formulation Pregnyl (Organon). This is an hCG containing drug which is manufactured from the purification of the urine of pregnant woman. The intact hCG heterodimer is the dominant hCG variant present in the drug, and is responsible for the pharmacological benefits obtained from an injection of Pregnyl. If present, the free hCG β-subunit also display some biological activity, but to a much smaller extent [52]. The Pregnyl formulation also contains hCG degradation variants, namely the two nicked hCG molecules and the hCG β-core fragment. These degradation variants do not display any relevant biological activity. Since the developed MS method detected both intact hCG and free hCG β-subunit through the same signature peptide (βT5) the detection of this signature peptide was related to the corresponding international units (IU) concentration of each Pregnyl ampoule.

When analyzing a digest of hCG (diluted in ammonium bicarbonate (ABC) buffer prior to proteolysis), it was observed that the peaks representing the detection of the nicked variants and the hCG β-core fragment were, as expected, substantially lower in signal intensity than the peak of the intact hCG/hCG β-subunit that related to the IU concentration. The use of Pregnyl as hCG standard has thus limitations in terms of quantification, as this will only be possible for the sum of intact hCG plus free hCG β-subunit. However, this formulation does contain the desired specter of hCG variants which reflects the hCG molecular diversity that might be present in a biological matrix, and was as such suitable for the LC-MS design and development of the method.

3.1.4 **Multiplexing hCG identification through LC-MS based detection**

The gradient used for hCG peptide mapping described in section ***3.1.2*** was 32 minutes long, resulting in a total analysis time of 50 minutes when including washing and regeneration of the analytical column. Further optimization of the LC gradient was thus performed in order to

achieve adequate separation of the signature peptides combined with short analysis time, and this resulted in a total analysis time of 27 minutes (**Paper II**). The specific retention time of all the signature peptides was thus established. Chromatograms are presented in following sections.

Combined with the dimension of retention time, the simultaneous SIM MS detection of various target hCG proteins through their diagnostic signature peptides could be performed (**Paper I**). Provided that adequate signature peptides are generated from any target proteins, this separation and detection of peptides using LC-MS offers a means of multiplexing the specific identification and differentiation of several proteins in one single run, as exemplified with the hCG molecules. However, whereas multiplexed detection of proteins is often defined as the simultaneous determination of *structurally different* proteins in one single run, this definition must be regarded with some caution in relation to the hCG molecules. This family of molecules is structurally related as they all share the hCG β-core fragment part. Furthermore, most hCG variants can be considered degradation variants of the intact hCG molecule. Nevertheless, the differences in molecular weight range from 15 kDa for the hCG β-core fragment to 37.5 kDa for the intact hCG. This justifies the use of the term "multiplexed detection" for the simultaneous and differentiated detection of these hCG molecules.

The acknowledged potential of LC-MS based multiplexing to be extremely specific and time-efficient makes this an interesting analytical technique for clinical biomarker analysis. Combined with an effective sample preparation strategy multiplexing of proteins in low abundance can be enabled.

3.2 Compatibility of immunoaffinity extraction with mass spectrometric detection

For the selective and specific extraction of target hCG molecules from complex matrixes adequate antibodies have to be carefully chosen. These will further have to be immobilized to a solid to enable isolation of the antibody-antigen complex that is formed during extraction. Following this, the biological matrix will be removed.

For this reason, the hCG specific monoclonal antibody E27 was selected as it is directed towards the core of the hCG β-subunit that is common to all hCG variants [35]. It will thus recognize and bind all hCG molecules. The antibody was immobilized to the walls of the wells in a 96 –well microtiter plate (**Paper I**), and serum samples containing spiked hCG were applied to the wells.

Immunoextraction of the target molecules was succeeded by extensive washing, followed by in-well reduction and alkylation. The subsequent addition of trypsin directly to the wells generated a peptide mixture that was subjected to a solid phase extraction (SPE) step prior to the final LC-MS analysis.

In the chromatograms resulting from these experiments the peaks of the signature peptides βT5, nβT5 44/45, nβT5 47/48 and cfβT9 eluted at the previously established retention time hereby demonstrating the successful extraction and detection of the hCG β-subunit (present in both intact hCG and as free hCG β-subunit), the two nicked hCG variants, and the hCG β-core fragment. For the detection of the hCG β-subunit linearity was observed for the concentration range of 100 to 2000 IU/L with a limit of detection (LOD) of 100 IU/L. The other hCG variants were qualitatively detected and differentiated, but as they were present in unknown and relatively low amounts no linearity or LOD were provided for these. The proof of the immuno-LC-MS principle was thus established, and this was further demonstrated by the analysis of serum samples from male patients previously diagnosed with testicular cancer. These experiments demonstrated the detection of intact hCG and free hCG β-subunit in a number of samples (n=20), in addition to the detection of nicked hCG in one particular sample. The varying β/α ratio in the different samples provided evidence that the free hCG β-subunit was present in addition to the intact hCG. Furthermore, the urine samples of pregnant women (at various stages in the pregnancy) were analyzed, although no prior experiments on urine samples had been carried out. The distinct detection of both the intact hCG/free hCG β-subunit and the main urine metabolite, the hCG β-core fragment, was demonstrated in all samples (n=6), indicating that the adoption of the developed immuno-LC-MS method to urine samples would not pose great problems.

What could be observed from the chromatograms of the serum analyses was that the signal-to-noise (S/N) ratio of the target peaks was relatively low compared to that of the peaks resulting from the analysis of the digest of hCG dissolved in buffer. This increase in noise at the base line strongly influenced the sensitivity of the method, and was explained by the unspecific binding of interfering serum proteins that were not removed by the washing steps. Although the washing procedure was optimized the level of noise remained relatively constant. It was therefore concluded that as the inference of serum proteins during immunoextraction clearly was problematic to exclude, other strategies had to be considered in order to increase the signal of the

target proteins and thus of the immuno-LC-MS based detection sensitivity. It was clear that the relatively limited sample capacity of the immuno-wells of 200 µL was not sufficient to provide adequate preconcentration of the target molecules to overcome the effect of the increase in noise following immunoextraction. As the LOD of the hCG method would need to be as low as 5 IU/L (a factor 20 lower than the current LOD of 100 IU/L), further improvement of the sample preparation strategy using this selective immunocapture had to be explored.

3.3 Optimizing method sensitivity and specificity

There are different ways to enable lower LODs for the immuno-LC-MS approach. Additionally, the use of complementary strategies will greatly enhance the effects of the individual strategy. For this reason the application of two different strategies was explored. First, it was assumed that a more selective and specific MS design would reduce the signal effect of the interfering co-eluting peptides on the detection of the target peptides (***3.3.1***). Secondly it was hypothesized that extraction of hCG molecules using antibodies coated to beads applied to larger sample volumes (***3.3.2***) would generate increased signal response of the target peptides through the increase of preconcentration factor (**Paper II**).

3.3.1 Tailored selected reaction monitoring design

By using a triple quadrupole detector in the SRM mode, MS detection can be performed enabling high structural specificity, sensitivity, and reproducibility. The transition from SQ detection to QqQ detection would as such provide a dual improvement to a method by decreasing the method's LOD and enhancing the analytical evidence for the identification of the hCG molecules.

The principle was employed to hCG, and for all hCG signature peptides specific SRM transitions were made based on the following criteria:

- The fragment ion signal intensity should be as high as possible
- Two or more diagnostic fragment ions should be included in the SRM transitions for each signature peptide
- The ratio of the two (or more) diagnostic fragment ions should be constant for every analysis performed under established conditions

The CID energy was thus optimized according to fragment ion intensity and abundance, and scan segments with optimized scan times generating at least six detection points per analytical peak were created.

With regards to quantitative measurements, an isotopically labeled internal standard was incorporated to the analysis to correct for intra- and inter-day variability produced by the MS. The selected internal standard AQUA peptide was an isotopically labeled synthetic analogue to the signature peptide βT5 of the hCG β-subunit, and was therefore named isβT5. This peptide contained an isotopically labeled arginine residue at the end of the sequence that resulted in a mass shift of +10 Da. In Figure 3.3 the detected mass difference of +10 Da between the selected fragment ions of the signature peptide βT5 and the isβT5 can be observed. The ratio of the fragment ions y11 and y8 was approximately 1:2, and constant (established ratio of 0.47 for n=20 and with RSD of 4%). The detection of the established abundance ratio of the diagnostic fragment ions increases the specificity of the method.

The AQUA peptide contained one cysteine residue that needed to be reduced and alkylated as to have the exact same physical-chemical proprieties as the signature peptide βT5. For this reason the labeled peptide is not a "classical" AQUA peptide; indeed the labeled peptide is calibrated prior to reduction and alkylation. The yield of alkylation thus has to be 100 % in order to ensure quantification accuracy. This was shown by full MS scan analysis in addition to tailored SRM analysis for both alkylated and non-alkylated labeled peptide. The alkylation yield proved to be 100 %.

Since the βT5 and the isβT5 are not separated in time, it is important that the diagnostic fragments ions used in the SRM program are y-ions. Although the precursor ions have different *m/z* values, the possibility of cross-talk phenomenon is present, as all b-ions generated do not contain the heavy arginine residue responsible for the mass shift in the fragments ions. Effects of cross-talk phenomenon were revealed by analyzing a test solution containing high amounts of isβT5 exclusively, and by using an SRM program in which transitions for both βT5 and isβT5 contained the identical b9-ion *m/z* value. From this experiment a response in the signal of the βT5 was demonstrated, although this peptide was not present in the solution. Cross-talk is most likely a theoretical problem, but in extreme cases such as samples containing high amounts of

isotopically labeled IS and no target protein/peptide, this could potentially have provoked a false positive response.

By increasing the selectivity of the detection, the LOD was extended from 100 IU/L (using MS detection) to 20 IU/L (using MS/MS detection). As such, the conversion from SQ-enabled SIM detection to QqQ-enabled SRM detection provided an increase in LOD by a factor 5. This approach included immunoextraction with antibodies coated to wells prior to MS detection. Although improved, the LOD had to be further extended.

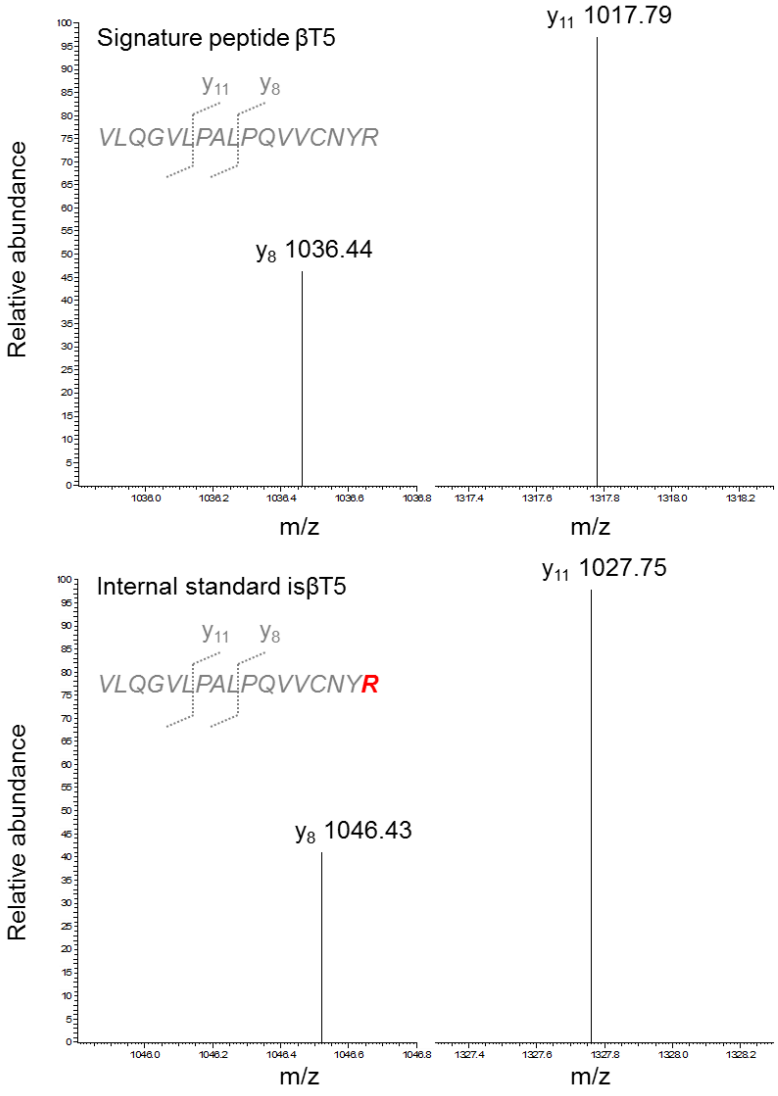


Figure 3.3 *Signals from the selected fragment ions of the signature peptide βT5 and the corresponding isotopically labeled internal standard, isβT5. For both peptides the amino acid sequence is shown, and the site of fragmentation for the selected y-ions is emphasized. The heavy arginine residue of the internal standard is visualized in red.*

3.3.2 **Immunoextraction using beads in stead of wells**

In order to explore the potential of increase in preconcentration factor as a function of sample volume, the antibody E27 was immobilized to magnetizable particles (beads) rather than wells. Different serum volumes were tested, and lowest LOD was obtained from the extraction of 1 mL serum sample. The resulting LOD was 5 IU/L, and although the sample volume was increased to 2 mL no further improvement in LOD was observed. Furthermore, as the amount of beads used for each extraction would determine the capacity of the method and as such define the linear range, varying amounts of beads were applied to 1 mL sample volume. For 20 µL of beads solution (10 mg beads/mL) linearity was observed for the concentration range of 5 to 5000 IU/L, compared to the range of 100 to 2000 IU/L, which was obtained when using antibodies coated to wells and SQ detection. All in all, the combination of selective MS/MS detection combined with immunoextraction using beads in stead of wells generated an improvement in LOD of a factor 20. The resulting chromatogram from the immuno-MS/MS analysis of the various hCG molecules spiked to serum is shown in Figure 3.4A, whereas that on the analysis of digested hCG diluted in buffer is shown in Figure 3.4B.

When comparing the two chromatograms in Figure 3.4 a second peak is observed in the cell of the hCG β-core fragment. Due to the difference in retention time this "interfering" peak does not compromise the detection of the authentic hCG β-core fragment signature peptide. Since this peak was not observed in the chromatogram of the digested hCG diluted in buffer, this must thus be an unknown peptide resulting either from interfering serum proteins or from the capture antibodies. The immunoaffinity extraction procedure using antibodies coated to beads is summarized in Figure 3.5. Since this procedure includes the reduction, alkylation, and tryptic digestion of the captured hCG molecules without releasing them from the antibody-bead complex first, the simultaneous trypsination of capture antibodies and unspesifically bound interfering serum proteins will also happen. However, interfering matrix analytes that are not completely removed during the wash procedure is a well-known problem of the immunoaffinity extraction strategy, and has to be overcome [34]. In this case this was accomplished through an increase in preconcentration factor combined with a selective MS detection SRM program.

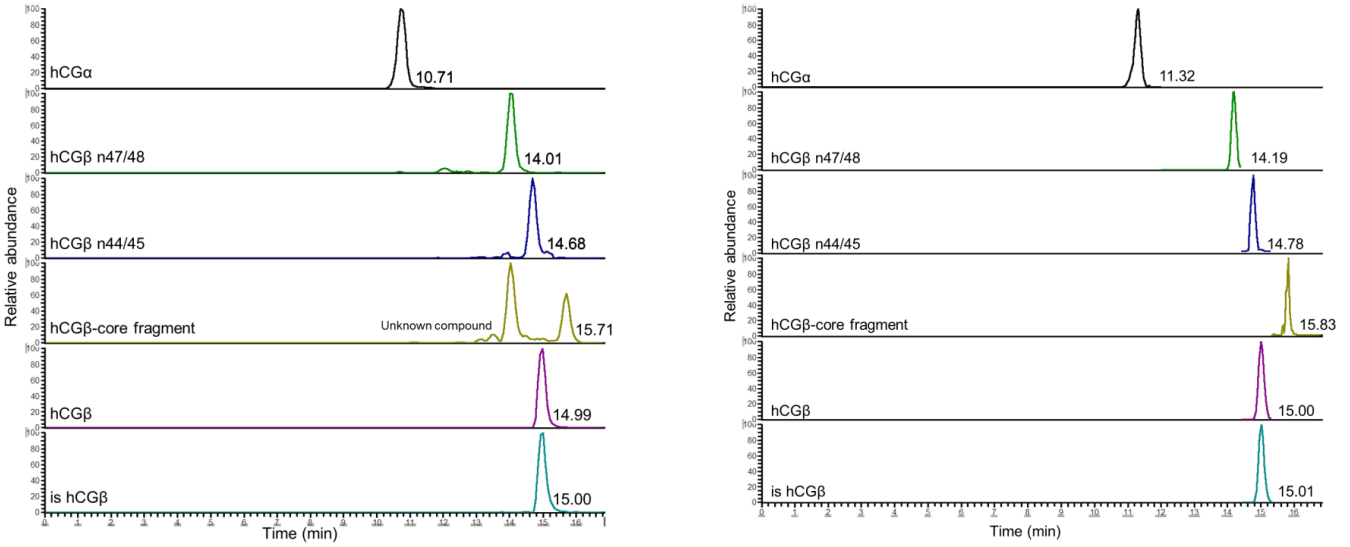


Figure 3.4 *SRM chromatograms showing the peaks of the different signature peptides and their respective retention times. The signal intensity is normalized, and the corresponding detected hCG variants are given for each signature peptide.* ***A)*** *The resulting chromatogram from the extraction, digestion and LC-SRM analysis of a serum sample spiked with hCG* ***B)*** *The resulting chromatogram from the digestion and LC-SRM analysis of hCG dissolved and diluted in buffer*

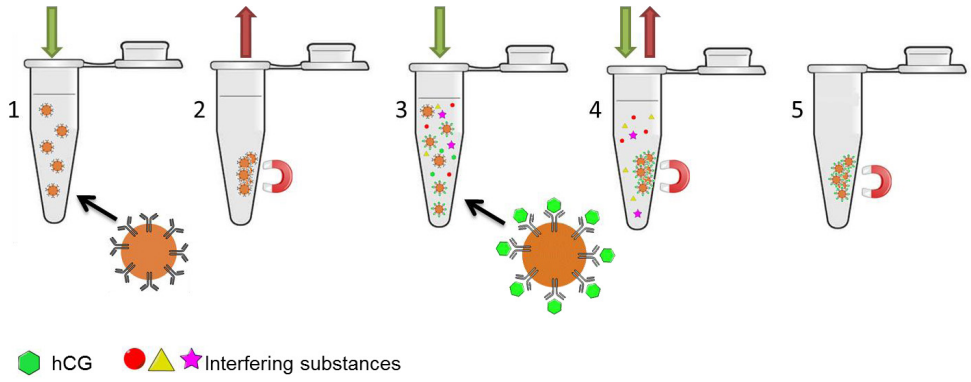


Figure 3.5 *The immunocapture procedure using antibodies immobilized on magnetic beads in stead of wells. The procedure can be summarized as follows: 1) Antibody coated beads are washed prior to extraction 2) Beads are isolated by magnet, and wash solution is removed 3) Sample is added, hCG bind to antibodies 4) Beads with antibodies and captured hCG are isolated by magnet. Interfering substances are removed by extensive washing 5) hCG has been extracted from serum, and will further be subjected to reduction, alkylation, and tryptic digestion directly in the tube without prior release from capture antibodies*

For other immunoaffinity extraction approaches combined with MS detection, other than the bottom-up approach, the release of target analytes from the capture antibodies is necessary prior to LC-MS analysis. This is often carried out using high molar concentrations of detergents, denaturants or acids that have to be removed prior to MS detection. This is time-consuming, and it is often seen that the recovery of analytes from the releasing procedure can vary to a great extent depending on the antigen-antibody interaction, hereby affecting the sensitivity of the method. The hCG bottom-up approach is not dependent on a pre-digestion release of the substrate as tryptic digestion of the entire antibody-substrate complex will occur (**Paper I**). However, it is possible that the dissociation of the antibody-antigen complex is necessary to obtain complete digestion, which is crucial for accurate quantification. Therefore, when the previously established reduction and alkylation step proved to dissociate the complex, there was thus no need to include an additional sample preparation step in order to release hCG from antibodies by other means.

The developed immuno-LC-MS/MS method for hCG determination, hereafter referred to as hCG immuno-MS method, had demonstrated a serum LOD of 5 IU/L. For quantification purposes, it had to be further established that the method generated reproducible, accurate and reliable results for every analysis. Additionally, the methods application to urine samples had shown great potential, and therefore had to be thoroughly tested.

3.4 Validation of method for quantitative determination of hCG

A thorough validation of a new methods analytical performance is essential for accurate quantitative analysis, and constitutes as such the foundation for implementation into routine analysis. The European Medicines Agency (EMEA) has defined a *Guideline on Validation of Bioanalytical Methods*, which defines key elements necessary for the validation of bioanalytical methods [98]. The hCG immuno-MS method was validated for both serum and urine (**Paper II**), and by using Pregnyl as standard the method was challenged to perform under realistic conditions as Pregnyl contains primarily the intact hCG molecule, but also smaller amounts of hCG degradation variants, as previously described in section ***3.1.3***. This implied that as quantitative measurements were provided based on the hCG β-subunit through the detection of signature peptide βT5, the sum of both intact hCG and free hCG β-subunit would constitute the quantitative response generated. Additionally, the presence of all other degradation hCG variants were simultaneously detected, although no validation data were sought for these as their quantitative amounts in Pregnyl are not known.

In **Paper II** all validation results were reported in international units (IU) since the clinical world is most familiar with hCG levels reported in IUs. However, in order to provide comparable sensitivity data that can be compared in a targeted proteomics setting, a conversion factor has been applied for translation of detected hCG β-subunit to molar concentration. According to Stenman (2006) will 1 IU of intact hCG correspond to 2.9 pmol [52], and since Pregnyl contains mainly intact hCG (and free hCG β-subunit to a much smaller extent), this conversion factor was chosen. It must be noted that a conversion factor the free hCG β-subunit also exist, but compared to intact hCG this molecule display a minor biological activity (1 IU free hCG β-subunit correspond to 42.5 pmol) [52].

The validation results for the quantitative detection of hCGβ using the developed immuno-MS method based on the targeted proteomics approach are summarized in Table 3.2. Furthermore,

the individual results are thoroughly discussed in **Paper II**. Sensitivity has long been the bottle neck preventing mass spectrometry from making the transition from discovery tool into the world of routine analysis. As can be deduced from these validation results, the successful cross-fertilization of the complementary techniques of immunoaffinity extraction and MS detection allows quantification of macromolecules in complex matrixes in the low picomolar ranges. In our research group this sensitivity-range has also been obtained for other clinically relevant tumor markers, such as neuron-specific enolase (NSE, Mw of 47.3 kDa, γ-subunit) and progastrin releasing peptide (ProGRP, Mw of 13 kDa) by using the bottom-up targeted proteomics approach [99]. This proves that the challenging procedure involving the enzymatically conversion of proteins into their constituent peptides can in fact be standardized to meet the criteria of bioanalytical validation guidelines. With this, mass spectrometry is brought one step closer to clinical diagnostics and routine laboratories.

For the validation of hCG it can be argued that the use of a proper international reference reagent (IRR) for the intact hCG molecule should have been used. However, satisfying validation was accomplished through the use of Pregnyl as standard although this posed more challenging conditions. Furthermore it must be noted that there are no obstacles for replacing the calibration and standard curves of the developed method with existing IRRs for hCG and hCG variants. The full quantification of all hCG variants was however beyond the scope of this project, since most clinical utility of hCG as biomarker today is based on the quantification of the intact molecule. Clinically relevant complementary information is nevertheless provided through the qualitative ratio generated through MS determination of other hCG variants.

Table 3.3 *Summary of validation parameters obtained for serum and urine samples. The LODs for serum and urine were estimated from a signal to noise (S/N) ratio of 3:1. The experimental LLOQs were defined as the concentration at which variation of less than 20% was obtained*

Validation parameter	Linearity (r^2) n=5	LOD n=5	LLOQ n=5	Selectivity (matrix) n=3	Selectivity (internal standard) n=3	Matrix effects
Serum	> 0.997	15 pmol/L 5 IU/L	29 pmol/L 10 IU/L	No interfering signal from blank matrix	No interfering signal from internal standard	No signal suppression/enhancement
Urine	0.999	6 pmol/L 2 IU/L	15 pmol/L 5 IU/L	No interfering signal from blank matrix	No interfering signal from internal standard	No signal suppression/enhancement

Validation parameter	With-in day precision (%) n=5		Between-day precision (%) n=5		Accuracy (%) n=5		Recovery (%) n=3	
Matrix	Serum	Urine	Serum	Urine	Serum	Urine	Serum	Urine
10 IU/L	19	9	13	14	95	104	39	65
100 IU/L	17	12	11	30	87	98	43	57
1000 IU/L	4	10	7	19	99	100	32	57

3.5 hCG immuno-MS in clinical diagnostics

A tailored biomarker immuno-MS tool can have multiple functions in a clinical scenario; it can be used to measure quantitative levels of well-established biomarkers in matrixes such as serum and urine; this will be demonstrated using the tailored hCG immuno-MS method for the analysis of clinical samples (***3.5.1***). Furthermore, the specificity of the MS analysis can be exploited to evaluate the performance and compatibility of other conventional immuno-based methods which are designed and tailored for defined pathological conditions. By characterizing the selectivity and specificity of various anti-hCG antibodies differentiating information regarding the various antibodies ability to recognize and isolate various target hCG analytes is provided, as will be presented below (***3.5.2***).

3.5.1 Pregnancy and cancer diagnostics

The validated method was used for quantification of hCGβ and differentiation between other hCG variants in serum and urine of a pregnant woman (**Paper II**). The serum hCG concentration was measured to 2.6 x 10^4 IU/L, which corresponded well with reported hCG levels at 21 weeks of pregnancy [52], and for the corresponding hCG concentration in urine was measured to 6280 IU/L. One of the resulting chromatograms is visualized in Figure 3.6, showing the detected hCG variants for both matrixes. The hCG α-subunit and hCG β-subunit were present in both matrixes, but the ratio of β/α was higher in the urine sample indicating that a proportion of the intact hCG had dissociated into free subunits, which is known to occur in urine. Additionally, the urinary degradation variants hCGβ-core fragment was observed in urine but not in serum, due to the well known degradation and excretion of intact hCG. For standard pregnancy testing it is obvious that this need not and should not be done by other means than standard pregnancy tests. However, for disorders of pregnancy the develop method can provide accurate quantification of the hCGβ (intact hCG and free hCG β-subunit), and hCG fingerprints differentiating the degradation variants (nicked hCG and hCG β-core fragment) from the unmodified hCGβ. It must also be noted that although eventual hyperglycosylated variants are not differentiated from the normally glycosylated variants, they are nevertheless captured and detected through the signature peptides of the protein backbone. Furthermore, since the free hCG β-subunit is an important marker for disorders of pregnancy, a more thorough exploration of the β/α ratio enabling differentiating quantitative measurements for both intact hCG and free hCG β-subunit, simultaneously, could result in increasing interest for the MS approach on this arena.

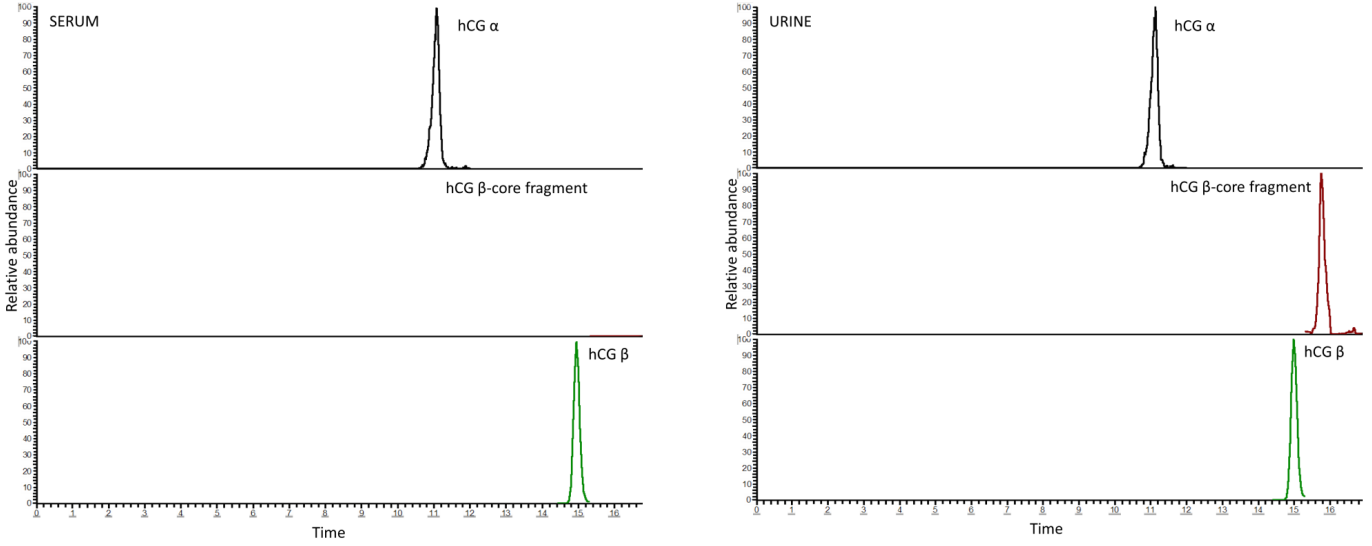


Figure 3.6 *Chromatogram of the analysis of serum leftt chromatograms) and urine (right chromatograms) samples from a pregnant woman (week 22), taken on the same day. The samples were extracted, digested and analyzed by LC-SRM using the developed tailored hCG immuno-MS method. The analysis showed detection of intact hCG and free hCG β-subunit in both matrixes, whereas the hCG β-core fragment was detected in urine exclusively*

The results from the analysis of the cancer patient serum samples generated quantitative measurements of this pathologically produced hCG that varied between ~200 and 20 000 IU/L. Additionally no other hCG variants other than the α- and β-subunit were observed. The chromatograms from some of these analyses are presented in Figure 3.7. In these chromatograms the hCG β-signals have been normalized whereas the α-signals are relative to the respective β-signals. It is also apparent that the β/α ratio vary from sample to sample, indicating that each sample contains various amounts of free hCG β-subunit in addition to intact hCG. This is supported by the literature that describes the free hCG β-subunit as an important tumor marker for certain types of cancer, although for many other types the intact hCG is often the dominant form. Other variants might also be produced for certain tumors, but these are mainly observed in urine as degradation variants of the intact hCG and the free hCG β-subunit [52,100-103,61].

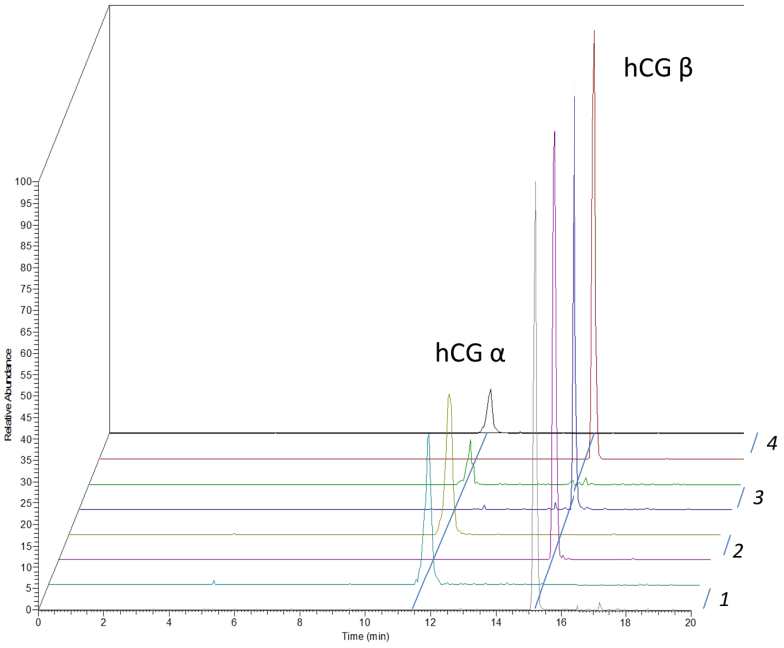


Figure 3.7 *Analysis of four serum samples of patients previously diagnosed with testicular cancer. The signal of the hCG β-subunit has been normalized whereas that of the hCG α-subunit is represented relative to the β-subunit. No other hCG variants were observed*

The potential of mass spectrometry to quantitate certain variants and differentiate others for use in a clinical setting has been demonstrated through the data presented on pregnancy and cancer samples. The method ensures that all hCG variants (both normally- and hyperglycosylated ones, heterodimers and free subunits) are detected. However, in a clinical setting this method should preferentially also enable the quantitative differentiation between intact hCG and free hCG β-subunit. This was also attempted, with partial success; following the establishment of the signature peptides for hCGα and hCGβ, a pure intact hCG standard was spiked to serum and extracted. This was done in order to establish the β/α signature peptide ratio for intact hCG when no free hCG β-subunit is present. The same ratio was to be established for defined increasing amounts of free hCG β-subunit added to the sample. However, for serum samples, adequate separation of the hydrophilic αT2 from other co-eluting hydrophilic compounds was not achieved, hereby preventing the absolute quantification of this substance (data not shown). These components were assumed to be interfering peptides resulting from the tryptic digestion of unspecifically bound serum proteins that were not removed during washing of the beads. This interfering signal could be ignored for high substance concentrations, but not for lower concentrations. Quite some effort was laid down on solving these chromatographic issues, but was not rewarded. For future prospects it was concluded that the use of nano-LC systems can be a suitable strategy for enabling improved separation. From the chromatograms of the urine sample, the same interfering signal was not observed. It was nevertheless decided not to explore this path any further since the quantification of the sum if intact hCG and free hCG β-subunit did not interfere with the principal scope of this project.

The possibility of tuning the selectivity of the method, in order to differentiate heterodimer from subunits, is most easily carried out through the selection of antibodies of known selectivity and specificity towards the different hCG variants [35]. This will limit the multiplexing feature of the method, but can be done if this differentiation is crucial for accurate hCG determination of certain clinical samples. The immuno-MS analysis will then have to be run several times for the same samples using the different antibodies. However, this is currently how the conventional immunoassays solve the differentiating issues.

3.5.2 **Evaluation of anti-hCG antibody selectivity and specificity**

The use of tailored MS detection to evaluate the selectivity of various anti-hCG antibodies to the different hCG variants is valuable in a clinical setting as this can provide information that is important for the tailoring of immunoassays towards specific biomarkers diagnostic of defined pathological conditions. This utility of the developed hCG MS detection was thus exploited to characterize various anti-hCG antibodies according to hCG epitope recognition (**Paper V**).

The International Society of Oncology and BioMarkers (ISOBM) initiated two Tissue Differentiation (TD-7) Workshops (WS) on hCG and related molecules. The project described in this thesis participated in the 2nd WS where 69 mAbs were tested for specificity and corresponding epitope recognition. From this antibody group, a panel of 30 mAbs was chosen for evaluation by MS (Figure 3.8).

International reference reagents (IRR) standards for intact hCG, nicked hCG, free hCG β-subunit, nicked free hCG β-subunit, hCG β-core fragment were spiked to separate bovine serum albumin (BSA) solutions, and extracted one by one using the 30 anti-hCG mAbs. For one mAb in particular, the hCG α-subunit IRR was tested since this mAb was known to be targeted towards the α-subunit. The detected signals were processed and presented in charts showing the various antibodies recognition of the different hCG molecules/standards, together with their classification according to specific epitope recognition. The outcome of these analyses is represented in Figure 3.8, where the results are compared to those obtained using a parallel method; the direct binding radioimmunoassay (DB-RIA) with ^{125}I-labeled hCG and hCG variant tracers.

For a majority of the antibodies the obtained MS data were highly concordant to the parallel study performed by DB-RIA, and supported thus the epitope group affiliation that had been previously suggested. The MS method can thus serve as a confirmatory method providing specific MS based verification of recognized hCG variants. Furthermore, also more complex data were obtained. These will be presented below.

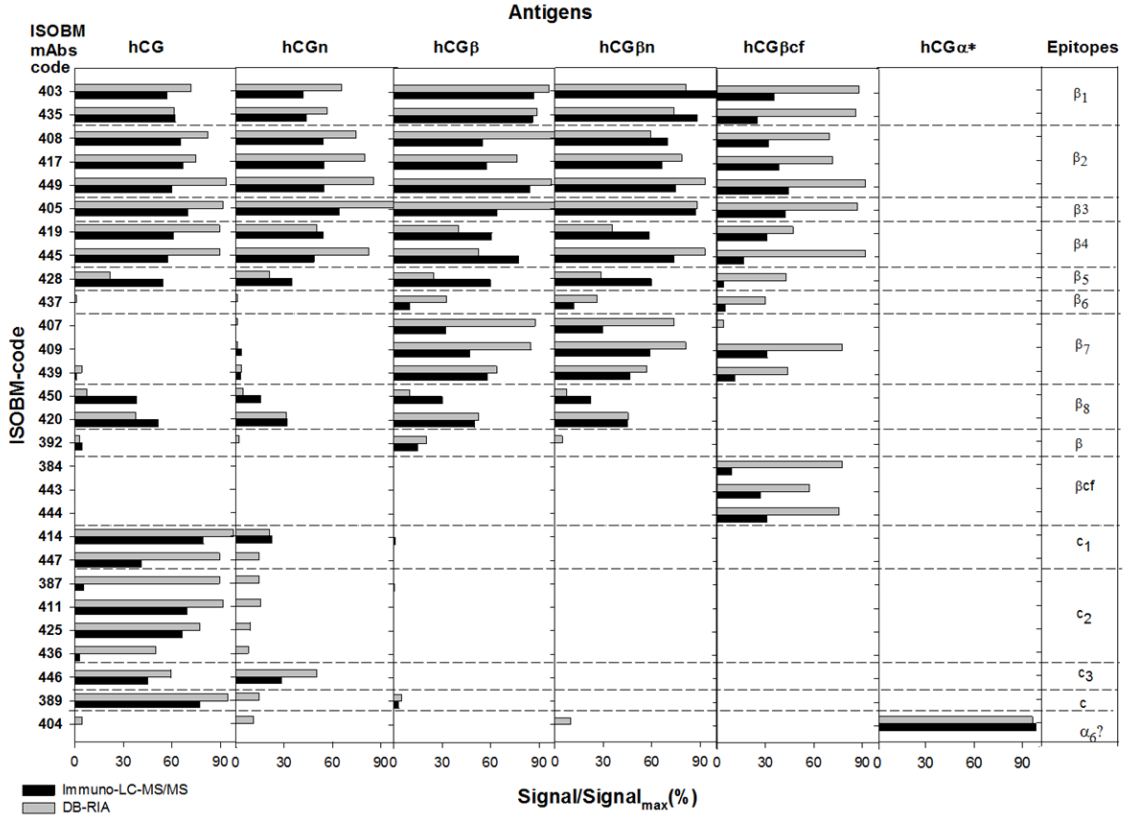


Figure 3.8 *Comparative classification of ISOBM-mAbs by DB-RIA and Immuno-LC-MS/MS. The antibodies are classified on the y-axis according to epitope group affiliation. The measured normalized MS signal intensity is represented by the ratio generated by the MS signal intensity of the detected hCG variant divided by that of the internal standard.*

Through the differentiating signal generated by the MS it became apparent that there existed a cross-contamination in one of the standards; the IRR of the nicked hCG heterodimer (99/642). Figure 3.9 shows the categorization of the tested anti-hCG mAbs and their respective recognition of the nicked hCG standard. In this figure the signals of the two nicked variants as well as that of the intact hCG cross-contamination are differentiated. From this chart it appears that the amount of intact hCG cross-contamination is approximately 20%. This correlates with what has previously been demonstrated by RP-HPLC analysis, indicating the presence of ~23.5% of nonnicked hCG [104]. As there is an apparent tendency for the antibodies that recognize intact hCG to recognize nicked hCG as well, this cross-contamination can be problematic for antibody-based assays that generate a signal that is the undifferentiated sum of all recognized hCG variants. However, the MS signal is not compromised as it clearly differentiates which part of the signal stems from authentic nicked hCG and which stems from cross-contamination.

Since a major criterion for the classification of hCG c-mAbs is the importance of nicks in the β-subunit, the ability of the MS to discriminate cross-contamination was particularly exploited in the characterization of these. Nicking in this area of amino acids number 44-48 disrupts some epitopes whereas others are unaffected. As can be seen in Figure 3.9 the MS showed that all signals elicited from the mAbs categorized in the c2-epitope group stemmed from the intact hCG cross-contamination. For some of these antibodies this had only been assumed previously, but was now demonstrated by the MS data (425, 387, 436). For others, this led to the precise sub-classification within this group (411). Furthermore the MS data showed that c3-mAbs recognize both nicked hCG and intact hCG (mAb 446), hereby contradicting the previous assumption that c3-mAbs are specific and selective for intact hCG.

There were however conflicting results observed as well; the c1-antibodies are also assumed not to recognize nicked hCG (such as mAb 447), and the mAb 414 had been thoroughly established as c1-antibody by other assays. The discordant results provided by the MS showed that this mAb did *de facto* recognize nicked hCG as well, leading to the conclusion that there appears to be an exception to the rule that c1-antibodes are specific for intact hCG. It could further be deduced that there seems to be some variability in the epitope cluster of the c-area, resulting in a continuum of c-epitopes with slightly different specificity patterns. The same tendency also exists for some of the β-epitopes (β2 to β6), but this knowledge has not been deduced from MS data.

One of the pit-falls of this MS based approach for characterization of antibody selectivity might be in the case of a signature peptide position being the same as for the current epitope. For two of the antibodies no signals were generated form the MS data whereas they had shown good signal responses in other assays. When looking at the suggested epitopes of these antibodies it appeared that they could very well be situated at the same location in the β-subunit as the signature peptides (amino acids 44-60 for hCG β, 47-60 for nicked hCG 47/48 and 45-60 for nicked hCG 44/45). It was thus hypothesized that the interaction between the capture mAb and the signature peptides for the target analytes might prevent trypsin from converting this part of the molecule to peptides, due to steric shielding. This has not been demonstrated.

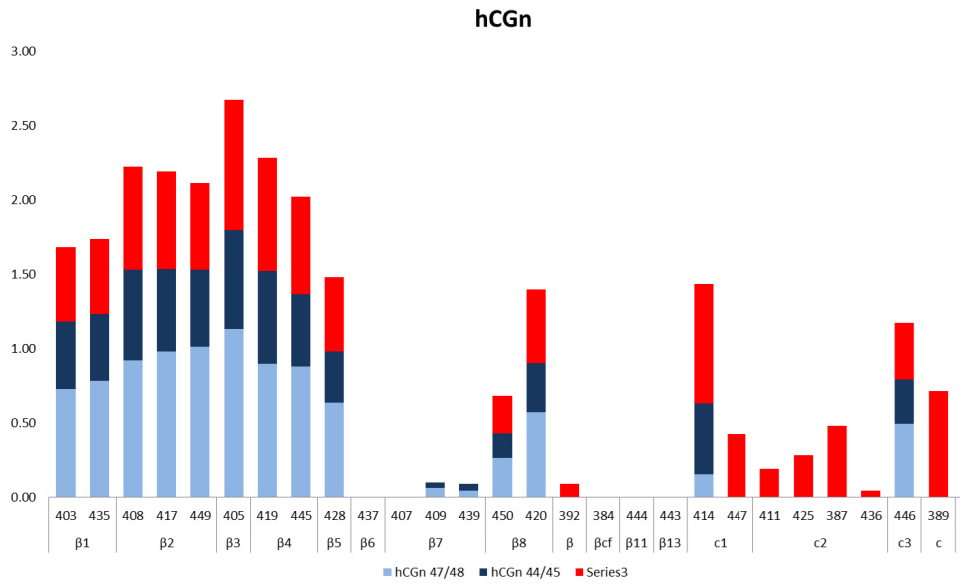


Figure 3.9 *Chart showing the various antibodies recognition of the nicked hCG. The antibodies are classified on the x-axis according to epitope recognition. The measured signal intensity (y-axis) is represented by the ratio generated by the MS signal of the detected target hCG molecule divided by that of the internal standard. The signal of the nicked hCG 47/48 is light blue, the signal of the nicked hCG 44/45 is dark blue, and the signal of the intact hCG cross-contamination is red.*

Assessing the analytical selectivity of mAbs through MS data offers an additional dimension to the analytical proof, which is sought through the well considered use of different methods for mAb selectivity evaluation. This is clinically relevant since it is crucial that assays tailored for defined clinical scenarios provide results that reflect the presence of the target analytes in the matrix, and not other structurally similar or otherwise interfering compounds.

3.6 hCG immuno-MS in doping analysis

In order to show the applicability of a developed method in a given analytical scenario, such as the arena of doping analysis, the method has to be challenged to perform on adequate realistic samples. For this reason a clinical study was initiated involving administration of hCG containing pharmaceuticals to males.

3.6.1 Clinical study

Through the conduct of a clinical study (**Paper III**) various aspects regarding detection of illicit hCG administration were to be studied, such as window of detection, differences in hCG concentration in serum versus urine, and molecular diversity of the hCG molecules as a function of matrix. The project was approved by the Norwegian Regional Committee for Medical Research Ethics (REK, http://helseforskning.etikkom.no), and a liability assurance was signed with the Drug Liability Association (http://www.laf.no). Furthermore, prior to entering the study each participant signed a letter of consent stating that they participated on a voluntary basis, were self-declared healthy, and were not competing athletes. Since hCG pharmaceuticals are currently available on the marked as both recombinant hCG formulations and urinary hCG formulations eventual differences related to type of hCG pharmaceutical were also of interest. Both variants were thus included in the study, and 12 healthy males received one injection of Ovitrelle (recombinant hCG, one dose corresponding to 6500 IU) whereas 12 others received one injection of Pregnyl (urinary hCG, one dose corresponding to 5000 IU). Serum and urine samples were collected prior to and following injection of drug to the abdominal fat tissue, for a period of fourteen days.

The window of detection was defined as the period of time for which hCG could be detected at concentrations above LOQ following injection of one single dose. In order to predict the window of detection for the two drugs theoretical pharmacokinetic elimination profiles were estimated for serum. This was based on the pharmacokinetic parameters provided by the SPC (summary of

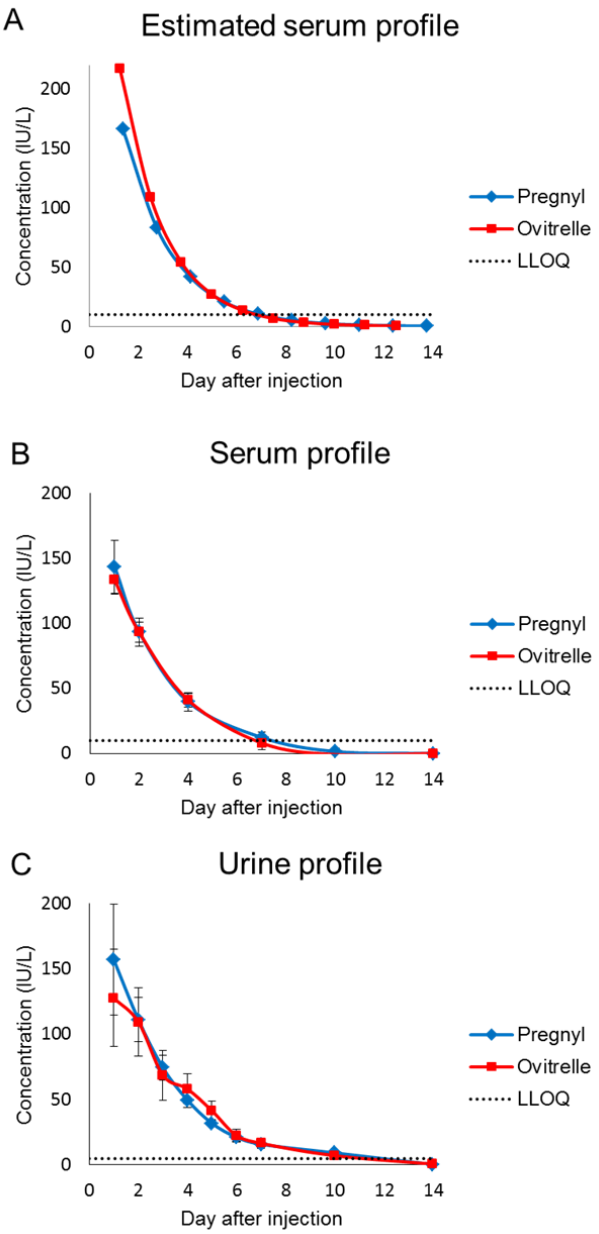
product characteristics) of the respective drugs. The resulting elimination profiles are shown in Figure 3.10A and from this a window of detection of 6-7 days could be deduced, for both pharmaceuticals. No estimation was made for the urine samples.

The experimentally derived hCG elimination curves for both serum and urine (Figure 3.10B and C) were produced based on the average detected hCG concentration (in the sample group) as a function of day following injection. The quantitative measurement was based on the detection of the hCG β-subunit. The elimination curve resulting from the analysis of the serum samples (Figure 3.10B) harmonized very well with the estimated curve, showing that the experimentally derived window of detection was, for both drugs, 7 days after administration of one single dose. Interpretation of the chromatograms further demonstrated that only hCG β- and α-subunits were detected in serum. Since hCG degradation variants, such as dissociated subunits, nicked and degraded variants, are mostly observed in urine this was not unexpected [52]. Furthermore, the presence of free hCG β-subunit in serum is mainly due to excess production of this subunit relative to the production of the α-subunit, and this process is related to the development of certain types of cancer [105,102,103,106,107]. The hCG β-subunit is further rapidly cleared form the circulation due to less efficient biological activity [55,52]. From this it was considered likely that the quantified hCG variant in serum was in fact the intact hCG molecule, and not the free hCG β-subunit.

For the urine samples (Figure 3.10C) hCG could be detected above LOQ (of 5 IU/L) until day ten following injection, generating a window of detection that is 3 days longer than what was shown for the serum samples. This was valid for both drugs. The broader window of detection observed for urine can be explained by the preconcentration of analytes in the morning urine samples. The chromatograms resulting from these analyses displayed the occurrence of hCG molecular variations through the detection of both nicked variants and the hCG β-core fragment in addition to the β- and α-subunit. The signal of these degradation variants was lower than the signal of the two main subunits, and in the samples taken on day five following injection the presence of the degradation variants could no longer be observed in the chromatograms. It was therefore concluded that the MS based detection of all hCG variants did not extend the window of detection for one injection of hCG containing drug. This was consistent with what has been reported in a previous study [108]. However, the repeatable injection of drug over a certain

period of time might alter the metabolic hCG trace and its respective window of detection. When comparing the respective molecular hCG pattern detected in the two groups having received Pregnyl and Ovitrelle, no unambiguous conclusions could be made allowing clear distinction between these. However a tendency towards more complex hCG variation could be indicated for the Pregnyl group.

The strategy of WADA to target the intact hCG is founded on targeting the relevant and active molecular form of hCG, and by doing so minimizing the possible association of the hCG finding with pathophysiological conditions. The ability to identify injected hCG would be optimal. Still, as there are no predefined hCG fingerprints that can exclude cancer as being the source (most cancer forms mainly produce intact hCG), this strategy of employing predefined method selectivity does not solve the clinical issues related to a positive hCG finding. Therefore, by giving a qualitative metabolic fingerprint in addition to the quantitative measurements more information is provided, possibly resulting in a direct benefit for the athlete affected. However, if the strategy of targeting the intact hCG molecule remains, this developed immuno-MS method can easily be tuned towards quantifying this molecule exclusively. By replacing the current antibody of broad selectivity and specificity towards all hCG variants by an antibody selective towards the intact hCG, the MS method is able to quantify the target exclusively. This way, the hCG immuno-MS method offers quantification at sensitivity as defined by WADA in addition to the specific identification of the relevant doping agent. This greatly reduces the risks of false positive and false negative responses associated with the conventional immunoassays.


A
Estimated serum profile
Concentration (IU/L)
200
150
100
50
0
0 2 4 6 8 10 12 14
Day after injection
Pregnyl
Ovitrelle
LLOQ
B
Serum profile
Concentration (IU/L)
200
150
100
50
0
0 2 4 6 8 10 12 14
Day after injection
Pregnyl
Ovitrelle
LLOQ
C
Urine profile
Concentration (IU/L)
200
150
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0
0 2 4 6 8 10 12 14
Day after injection
Pregnyl
Ovitrelle
LLOQ

Figure 3.10 *(On page 62) Pharmacokinetic elimination profiles of serum and urine hCG A) Estimated profile for Pregnyl and Ovitrelle in serum, produced on the basis of the pharmacokinetic parameters of the drugs B) Detected profile in serum using immuno-MS C) Detected profile in urine using immuno-MS. The profiles in B) and C) are produced based on the average detected hCG concentration as a function of day following injection. The variation displayed by the error bars is presented as standard error of the mean ($SE_{\bar{x}}$). For all plots: The y-axis hCG concentration refers to the sum of the intact hCG molecule plus the free hCGβ-subunit. The Pregnyl curve is marked in blue, the Ovitrelle curve is marked in red. For serum: LLOQ is 10 IU/L. For urine: LLOQ is 5 IU/L*

3.6.2 Comparison of hCG immuno-MS method to immunometric assay

An important aspect regarding a newly developed methods potential implementation to routine analysis is the comparison of its performance to one of the reference methods that is currently used by routine laboratories. For a MS based method this comparison will often be towards the conventional immunoassays, as these are widely used due to their high throughput, sensitivity and cost-efficiency.

In doping analysis the hCG immuno-MS method showed potential as confirmatory method following a positive hCG finding in the screening phase. Its comparison was thus carried out towards one of the confirmatory immunometric assays currently used by WADA accredited laboratories, the DELFIA® hCG assay [109] (**Paper IV**).

A selection of 149 samples from the available biobank of the clinical study constituted the foundation for the comparison. These had already been analyzed with regards to hCG content by the hCG immuno-MS method, and the outcome had been described in **Paper III**. At the time of the analyses performed by the DELFIA assay the samples had been stored at -32˚C for six additional months, and had been subjected to an additional cycle of freezing and thawing. Admittedly, this is not ideal when comparing method performance but at the time being it was what was at hand, and the idea was to get an indication of the methods performance compared to a reference method, in terms of quantitative measurements and window of detection.

Whereas stability issues related to urine hCG diversity complicated the interpretation of the comparison between the methods, the interpretation of the serum results was relatively straight forward. As can be seen in the Bland-Altman plot in Figure 3.11A good agreement between the two methods was observed, with a correlation coefficient of 0.0096. This was further supported by the superposed serum hCG elimination curves produced on the basis of average detected hCG values for the two methods (as a function of days following injection of hCG), as shown in Figure 3.12A.

The window of detection was here defined as the number of days for which quantification of illicit hCG levels is possible after injection of a single dose of hCG. What could also be deduced from the elimination curves in Figure 3.12A were comparable windows of detection for the two methods. The consensus that thus was derived from the serum hCG measurements proved the agreement between the two methods and hereby their ability to generate comparable serum hCG responses. This consensus was most likely achieved in serum due to the relatively stable and homogenous content of intact hCG, that would not provoke any differences in measurements as a function of method selectivity.

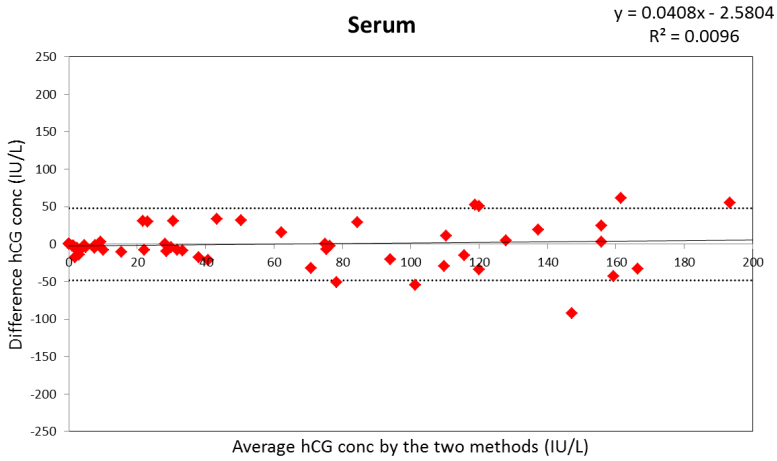
For the urine measurements a systematic difference could be deduced from the proportional error observed as a positive linear slope above the mean. When comparing the biological hCG elimination pattern produced by the two methods (Figure 3.12) lower measurements were reported by the DELFIA assay explaining the observed difference from the Bland-Altman plot. This also affected the window of detection, which was two days shorter than what was reported by the immuno-MS method.

The discrepancies in the urine measurements were attributed to deterioration of the intact hCG content in urine during storage combined with the difference in selectivity of the two methods. The dissociation of intact hCG heterodimer into its free subunits in urine upon storage has been studied and described elsewhere [110,111]. As the DELFIA assay targets the intact hCG whilst discriminating all other hCG variants including the free hCG β-subunit will this heterodimer dissociation clearly affect the measurements provided. Since the urine samples had been stored for a much longer time than what is normal in a doping analysis scenario and further since the analyses were not performed at the same time for the two methods, no conclusion could be drawn for the urinary hCG window of detection. However, the immuno-MS method circumvents the

potential problem of heterodimer dissociation as it quantifies the sum of the free hCG β-subunit and the intact hCG, hereby avoiding reporting falsely low intact hCG levels.

Since agreement of the methods was demonstrated for the serum measurements, as well as comparable windows of detection, it is likely that this agreement would be valid for urine measurements as well. This is provided that the methods are allowed to perform on the same sample material containing the same hCG content, i.e. that the samples have been subjected to the same time of storage and the same processes of freezing and thawing. The broader selectivity of the immuno-MS method enabling quantification of both intact hCG and free hCG β-subunit might even allow a longer window of detection since a certain amount of intact hCG is likely to dissociate in urine during shorter storage times as well. Nevertheless, the specific identification of target analyte will only be provided by the MS-based method.

A



B

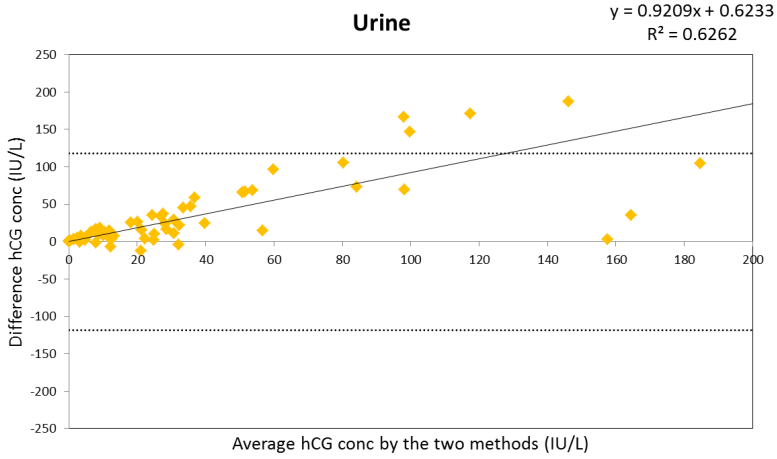
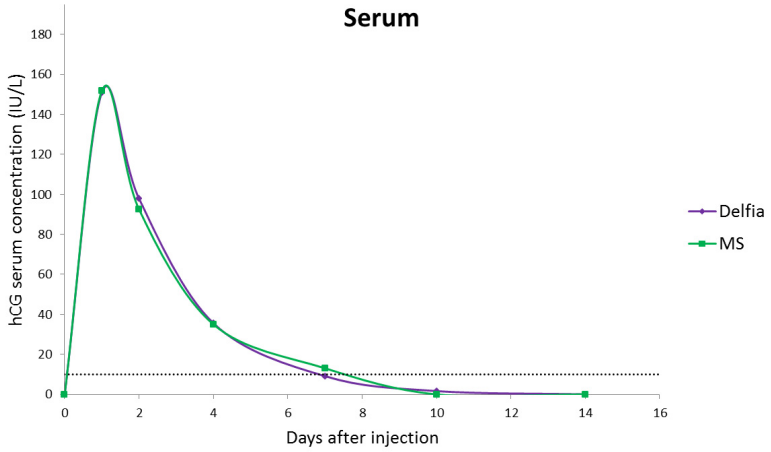


Figure 3.11 *Bland-Altman plots showing the agreement between the DELFIA assay results and the MS method results from the analysis of A) the serum samples and B) the urine samples. The average detected hCG concentration by the two methods is given on the x-axis, and the difference reported by the two methods is represented on the y-axis. The limits of agreement are specified as bias ± 1.96 STD (average difference ± 1.96 standard deviation of the difference.*

A



B

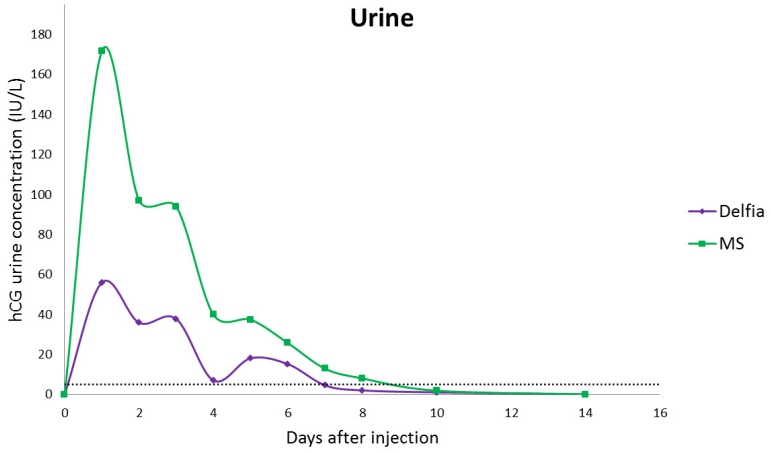


Figure 3.12 *The average detected hCG concentrations generated by the two methods as a function of day following injection, in A) serum and B) urine. The average concentration is provided from the analysis of ~12 samples per point in the serum curve, and ~8 samples per point in the urine curve. The curves representing the results of the analysis performed by the DELFIA assay are purple, whereas those of the MS method are green.*

3.7 Future perspectives

LC-MS/MS has experienced substantial growth in clinical laboratories during the last 10-15 years, but this has been limited to the analysis of smaller molecules due to the challenges related to MS-based peptide/protein analysis [25]. The advantage of mass spectrometry is first and foremost the strength of the analytical evidence related to the absolute identification of the target analytes. Problems related to false positive responses and the accompanying consequences for the patients are thus eliminated. Furthermore the ability to multiplex and differentiate the simultaneous detection of several target analytes through the dimension of LC separation has shown great potential in terms of time- and cost-efficient screening procedures, and the advantages related to multi-biomarker profiles tailored for various diseases is undisputed [25]. This applies for diagnostic utility but also for monitoring of disease development and response to therapy. In a future where tailored therapeutics for the individual patient is aimed for, it will be of utter importance that the analytical techniques employed to evaluate the effects of the pharmaceutical interventions provide detailed and differentiating data of the pathological status quo. For this to be feasible in clinical laboratory every-day-life, the clinical results have to be achieved the easiest and most reliable way possible. The presented results have partially shown the clinical application of this targeted immuno-LC-MS/MS approach to the hCG molecules.

For the hCG immuno-MS method to fulfill its potential for optimal use in diagnostics and disease management, the sensitivity has to be extended even further since the reference hCG concentrations in men and non-pregnant women is as low as 0.7-5.4 IU/L [52]. This can likely be achieved by converting to nano-scale LC-MS/MS, where the main advantage of using smaller i.d. columns in HPLC is the increased detection sensitivity that can be obtained as a result of reduced sample dilution. In addition, the increased efficiency of ionization that results as a function of the low flow rate further extends the sensitivity of the analysis. As such the main application of nano-LC is the separation of biomolecules in proteomics and biotechnology. Such an instrumental set-up is however fairly complicated to manage and less robust, and requires thus a great level of expertise from the operators. This might consequently hamper the implementation of hCG-MS into a clinical setting, and the benefits must be weighted compared to the disadvantages.

To further improve the methods applicability in a clinical setting, a natural progression would be to use the 1st IRR standards to enable the quantification of all hCG variants. With regards to this it could be of interest to explore other quantification strategies, to overcome economical and practical issues related to the multiplexed quantification of several hCG variants in one run. A QconCAT standard that includes all hCG signature peptides is a relevant alternative. This standard will provide equimolar concentrations of the various signature peptides and thus do not require quantification of each standard separately. As such the QconCAT is well suited for multiplexed strategy, and decreases costs whilst increasing throughput.

Additionally, and as pointed out in the discussion in section 3.5.1, the quantitative differentiation between heterodimers and free subunits should also be enabled. The establishment of the suggested β/α ratio is a cleaver solution to this problem, provided that there are no other heterodimer variants (nicked variants) present in the sample. The latter might actually confuse the interpretation of data regarding the belonging of the α-subunit; does it stem from intact hCG or nicked hCG? Therefore a more robust differentiation can be achieved through the use of mAbs selective for the heterodimers exclusively, namely c-mAbs (their selectivity is shown in Figure 3.8). This will require two analyses for the same sample, but will generate robust and reliable data.

In modern sports drug testing, the development of MS based strategies for detection of the continuously emerging peptide- and protein-based pharmaceuticals is likely to present a rapid and solid alternative to the time-consuming development of antibody-based methods. In its most effective extent this comprises what has in part been demonstrated by the developed hCG immuno-MS method; the simultaneous screening of a group of different proteins in one single run. As the luteinizing hormone is also prohibited for males whilst in competition, the developed immuno-MS method could gain effectiveness and interest by incorporating the detection of this structurally similar hormone in the same multiplexed run.

CONCLUDING REMARKS

The presented thesis describes how the targeted proteomics strategy has been utilized in combination with immunoaffinity extraction to enable sensitive and specific MS determination of various hCG proteins in one multiplexed run. Through immunoaffinity extraction of target proteins the MS has been granted access to the low abundance target proteins, enabling unsurpassed LOQs for MS determination of macromolecules from complex matrixes such as serum and urine. As such the presented results argue the potential of immuno-MS to have a future in clinical chemistry, and thus make the transition from discovery tool to routine analysis.

The unmet specificity of the MS data is particularly valuable for biomarker identification in different arenas such as routine clinical diagnostics and in doping analysis, but also for experiments assessing antibody selectivity. Additionally, there is currently a critical demand for rapid and robust methods to evaluate the vast number of potential biomarkers emerging from whole proteome experiments, carried out by different shotgun proteomics strategies. This biomarker validation requires feasible, time- and cost-efficient analysis of a limited number of candidate proteins on a large number of samples. As shown in the presented work, the multiplexing immuno-MS method can enable high throughput through the simultaneous determination of several low abundance proteins.

The surrogate detection of a unique structural component in the place of a macromolecule makes the targeted proteomics approach by SRM MS detection exquisitely specific and sensitive, and this detection technology is highly compatible with accurate quantification strategies based on the SID principle. As shown in this work the combined used of an AQUA peptide and an external standard has allowed accurate quantification of proteins from complex proteomes at picomolar levels. It is thus argued that targeted proteomics based methods can be designed in a standardized and reproducible manner to facilitate genuine comparison of data between laboratories. Furthermore, by using commercially available reagents that do not require particular in-house expertise, methods can be implemented in a harmonized manner across different laboratories.

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IV

Epitope Analysis and Detection of hCG Variants by Monoclonal Antibodies and Mass Spectrometry

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ABSTRACT

The reactivity of 30 monoclonal antibodies to six hCG variants was measured using an immunoselection technique combined with LC-MS/MS detection. Each of the antibodies had been characterized with regard to epitope specificity during the 2nd TD-7 Workshop. For most mAbs directed against hCGβ the MS data was highly concordant to that obtained using the standard immunological methods employed during the workshop. The ability of MS to simultaneously perform epitope analysis and identification of the hCG variant facilitated the detection of intact hCG cross-contamination in the international nicked-hCG 99/642 standard. This led to fine-tuning of the previous sub-classification of the heterodimeric hCG-reactive c-mAbs; c_2 mAbs did not recognize hCGn as the signal observed for the hCGn standard was strictly that of the cross-contamination. Furthermore, evidence was obtained for c_3-mAb recognition of both hCG and hCGn. One mAb, belonging to c_1-mAb subgroup, was unique, in being able to recognize nicked as well as intact hCG. Complementary MS-based analytical evidence thus proved useful in the characterization of anti-hCG mAbs, contributing to their precise classification according to epitope recognition.

INTRODUCTION

Human Chorionic Gonadotropin (hCG) is a placental hormone produced by trophoblastic cells during the entire course of pregnancy and serves as a biomarker for pregnancy detection (1). It is also used clinically as a marker for disorders of pregnancy (gestational trophoblastic diseases) (1), nontrophoblastic neoplasms (i.e. germ cell tumors) (2-6) and Down syndrome screening (7).

The term hCG describes a heterogeneous group of variant molecules. Intact hCG, is a heterodimeric glycoprotein (37.5 kDa) which is composed of two subunits; hCGα (92 amino acids, 14 kDa) and hCGβ (145 amino acids, 23.5 kDa). The subunits are non-covalently associated and internally stabilized by a cystine knot consisting of three disulfide bonds (8, 9). From this central cystine knot two beta-sheet protein loops (1 & 3) protrude from one side. They are opposed by the single larger loop 2. Structural differences in hCGβ result in variants of the hCG molecule. These variants comprise free hCGβ subunit, nicked hCG (hCGn) and nicked hCGβ (hCGβn) which are both cleaved between hCGβ amino acids 44/45 or 47/48, metabolic variants like hCGβ core fragment (hCGβcf) comprising amino acids 6-40 and 55-92, and differentially glycosylated variants (10, 11). The heterogeneity of related hCG molecules challenges precise hCG detection; what lies within the total hCG response that is provided by tradiotional used immunometric assays? Clearly, not all anti-hCG antibodies (Abs) recognize the same hCG variants, or to the same extent.

Monoclonal Abs (mAbs) against hCG can be classified according to their major subunit specificities. (i) mAbs that recognize free and/or assembled hCGβ and derivatives (hCGβ-mAbs), (ii) mAbs that recognize free and/or assembled hCGα (hCGα-mAbs) and (iii) mAbs that bind to heterodimeric hCG (c-mAbs) but do not recognize free subunits or variants thereof.

The International Society of Oncology and BioMarkers (ISOBM) initiated two Tissue Differentiation (TD-7) Workshops (WS) on hCG and related molecules. The 1st TD-7 WS comprised 27 mAbs (11), whilst the 2nd TD-7 characterized the epitope specificity of 69 mAbs, (reported back-to-back in this issue,).

Mass spectrometry (MS) has emerged as a state-of-the-art technique for in-depth characterization of therapeutic antibodies, and is being used at all stages of mAb production, and preclinical and clinical mAb development (12). Additionally, valuable insights in structure-function relationships have also been gained (12). However, a complementary dimension of MS utility in relation to mAbs is the MS based characterization of mAb selectivity and specificity (13). Specific and tailored hCG detection based on mass spectrometry following the extraction of five different hCG variants by 30 anti-hCG mAbs enables differentiating data regarding the various capture Abs ability to recognize and isolate target hCG analytes. This differentiated MS response complements the uniform response representing the sum of all captured and detected molecules provided by other conventional immunometric detection methods.

This targeted proteomics approach using MS for detection of hCG and related molecules is based on enzymatic cleavage of proteins into their constituent peptides. The peptide mixture is subsequently separated using liquid chromatography (LC) prior to MS/MS detection. Unique signature peptides are generated which are characteristic for their parent hCG proteins and related molecules (14, 15), and these signature peptides are represented in figure 1 along with their corresponding parent protein. Specific and unique mass fingerprints for each hCG variant enable the reliable identification and simultaneous differentiation between the captured molecules (14). This method thus provides evidence for which hCG variants are recognized by the various capture anti-hCG Abs. Finally, and perhaps most importantly, the MS detection will can reveal

78 impurities in the standards, i.e. cross-contamination of other hCG variants in the current standard.
79 In this paper, we used LC-MS/MS to comparatively characterize 30 anti-hCG mAbs from the 2nd
80 ISOBM workshop with respect to their recognition of intact hCG, hCGn, hCGβ, hCGβn and
81 hCGβcf.

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MATERIAL AND METHODS

Monoclonal antibodies

Sixty nine mAbs were submitted by eight different participants to the scientific coordinator of the ISOBM workshops. The mAbs were coded and forwarded to the working groups. A panel of 30 anti-hCG monoclonal antibodies (table 1) was used for immunoextraction and liquid chromatography tandem mass spectrometry (LC-MS/MS) experiments. Different mAbs were chosen to represent the different epitope groups.

Reference reagents for hCG and related molecules

Reference reagents for hCG were obtained from the National Institute of Biological Standards and Controls (NIBSC). These included the 1st International Reference Reagents (IRRs) for intact hCG (99/688), hCGn (99/642), hCGβ (99/650), hCGβn (99/692), hCGβcf (99/708) and hCGα (99/720). The 1st IRRs were assigned values in substance concentrations (mol) (16, 17). The lyophilized content of each ampoule was reconstituted with the appropriate volume of phosphate buffer saline (PBS) containing 10 g/L BSA, generating a concentration of 1 nmol/mL for each IRR. Each preparation was aliquoted and stored at -20 ˚C.

Coupling of antibodies to magnetizable particles

Coupling of antibodies to magnetizable particles was performed essentially as previously described (18) using 20 mg of antibody to 1 g of Dynabeads (dynabeads M280 tosylactivated, Life Technology, Invitrogen Dynal, Oslo, Norway). This resulted in approximately 15 µg of immobilized antibody/mg of bead suspention. The solution was diluted to 10 mg of beads/mL using PBS containing 0.1 % BSA (pH 7.4).

Immunoaffinity extraction

The immunoaffinity extraction procedure was performed essentially as previously described (14). Each hCG IRR aliquot was thawed and diluted to 0.5 pmol/mL, (except for IRR 99/708 which was diluted to 5 pmol/mL) using 50 g/L BSA in PBS?. In the experimental set-up for the evaluation of the performance of the 30 monoclonal antibodies, triplicates of 1 mL sample volume were performed (n=3) for each extraction of the respective IRRs. All antibodies were tested for specificities towards five 1st IRRs (99/688, 99/642, 99/950, 99/692 and 99/708), except for mAb 404 for which also the IRR for hCGα (99/720) was included.

The antibody-coated beads (20 uL per extraction) were washed using 500 uL PBS containing 0.005% Tween 20 prior to extraction. To facilitate antigen-antibody interaction the samples were placed in a HulaMixer (Invitrogen) for 1 h. Then the beads were washed three times with PBS (0.005% Tween 20; 500 uL), PBS (500 uL) and finally 10 mM Tris-HCl (pH 7.4). A magnetic rack was used for isolation of the magnetic beads whilst removing wash solution from the eppendorf tubes. l

Tryptic digestion

Freshly prepared 50 mM ammonium bicarbonate buffer (200 uL) was added to the tubes containing the antibody beads with the captured hCG molecules. Reduction was performed with dithiotreitol (DTT) prior to alkylation with iodoacetic acid (IAA), followed by addition of trypsin to the tubes, as described (14). The beads were not removed from the tubes. Tryptic digestion was performed at 37˚ C for 18 hrs with gentle agitaion. The peptide digests were then subjected to solid phase extraction (SPE), and the beads discarded. Evaporation of the eluate and further reconstitution of the dried peptides in 20 mM formic acid followed the SPE (14).

Liquid Chromatography-Mass Spectrometry

The samples were loaded on the liquid chromatographic mass spectrometric (LC-MS/MS) system for separation of peptides using reversed phase chromatography, followed by MS detection of preselected signature peptides. The signature peptides representing the various hCG molecules are schematically represented in figure 1 along with their corresponding parent proteins.

The Dionex UltiMate 3000 chromatographic system consisted of an autosampler, two pumps, and a flow manager, all Dionex (Sunnyvale, CA, U.S.A.). The processing was managed through Chromeleon software, version 6.80 SR6. The triple-quadrupole MS detector (TSQ) was a Thermo Scientific TSQ Quantum Access detector. Data acquisition and processing were carried out using Xcalibur software, version 2.0.7 SP1. The chromatographic separation was carried out on a Biobasic-C8 column from Thermo Scientific (Rockford, IL, U.S.A.) as described earlier (14). The electrospray ionization (ESI) source was operated in the positive ionization mode, and experiments were performed in the SRM mode, using predefined specific *m*/*z* values for hCG signature peptides (14, 15).

Method validation

The analytical sensitivity was determined to be 5 IU/L. The validation of precision indicates a total variation of between 4 and 19 % for concentrations of 10, 100 and 1000 IU/L. All samples were analyzed in triplicate.

RESULTS AND DISCUSSION

MAb specificities towards the 1st IRRs for hCG and variants

The detected signal responses for each hCG variant recognized by the ISOBM-mAbs are summarized in figure 2A-E. For a majority of the antibodies the LC-MS/MS data are highly concordant to the parallel study performed by direct binding radioimmunoassay (DB-RIA) with ^{125}I-labeled hCG and hCG variant tracers (reference), and thus supports the epitope group affiliation that has been previously suggested (reference). The following mAbs (25/30) have been reliably epitope typed: epitope β_1 (ISOBM-403 and -435), epitope β_2 (ISOBM-408, -417 and -449), epitope β_3 (ISOBM-405), β_4 (ISOBM-419 and -445), β_5 (ISOBM-428), β_6 (ISOBM-437), β_7 (ISOBM-407, -409 and -439), β_8 (ISOBM-450; type 1 and -420; type 2), βcf (ISOBM-384), β_{11} (ISOBM-444), β_{13} (ISOBM-443), c_1 (ISOBM-414 and -447), c_2 (ISOBM-411, -425, -387 and -436) and c_3 (ISOBM-446). For the following antibodies, no specific epitope sub-classification has been established: hCGα-mAb ISOBM-404 (α_6?), hCGβ-mAb ISOBM-392, and c-mAb ISOBM-389. No signal was generated in LC-MS/MS for ISOBM-386 and -422.

hCGα-mAbs

According to DB-RIA ISOBM-404 is a specific mAb for non-assembled hCGα. Although the exact molecular epitope localization has not been investigated using synthetic peptides it is assumed that ISOBM-404 is directed against epitope α_6 (amino acids hCGα 33-41) since this is the only region of hCGα that is known to elicit high affinity Abs specific for free hCGα. As can be seen from figure 2F the complementary information retrieved from the LC-MS/MS data confirms that ISOBM-404 *de facto* is specific for hCGα and does not recognize hCG or hCGn (Fig.2F).

hCGβ-mAbs

Most mAbs directed against hCGβ have been reliably epitope typed using techniques like DB-RIA (see accompanying paper). The LC-MS/MS data confirmed these classifications (see above). MAbs against epitopes $\beta_{1\text{-}5}$ recognized hCG and the four hCG/hCGβ related variants, whereas mAbs against epitopes β_6 and β_7 predominantly recognized free hCGβ, hCGβn and hCGβcf. MAbs against the linear epitope $\beta_{8,}$ which occurs at the very end of the hCGβ specific carboxyl-terminal peptide (CTP), recognized all hCG variants except for hCGβcf. MAbs targeted against epitopes β_{11}, β_{13} and βcf all recognized hCGβcf exclusively.

LC-MS/MS confirmed the non-involvement of hCGβ loop 2 in epitopes β_1-β_5 and the CTP-located epitopes as neither nick 44/45 nor 47/48 influenced binding of the mAbs (Fig3). However, a few uncertainties related to epitope sub-classification remained. The LC-MS/MS data showed, that in accordance with the DB-RIA results, ISOBM-392 predominantly recognized hCGβ and to a minor extent intact hCG. Interestingly the LC-MS/MS signal generated by using immunoaffinity extraction of hCGn originated from the cross-contamination in the 1st IRR with intact hCG. Thus this mAb is targeted against hCGβ, however, the specific epitope localization remained unresolved. Signal responses generated by ISOBM-392 were fairly low as observed in the LC-MS/MS, the DB-RIA methods and affinity analyses by Forster Resonance Energy Transfer (FRET; see also accompanying publication, Ref). Thus ISOBM-392 displays low affinity thereby limiting the sensitivity and the significance of data by any method. Final epitope characterization of this mAb seems to be of minor relevance due to its moderate to low affinity, and it is thus unlikely that this mAb will be clinically useful.

Furthermore, some disparity of the LC-MS/MS data as compared to the DB-RIA results was observed regarding mAb ISOBM-407. In sandwich assays this mAb been grouped with Abs that

recognized the cystine knot related epitope β_7 (reference), although in DB-RIA it showed lower cross-reactivity with hCGβcf (4%) as compared to other β_7 mAbs (ISOBM-409 and -439). The LC-MS/MS data revealed no reactivity with hCGβcf for this mAb (Fig.2E). However, the lack of signal in the MS can be explained by the low signal for the hCGβcf in LC-MS/MS. This is due to poor extent of ionization in the electro-spray which greatly influences the signal intensity and thus method sensitivity, and is the reason why all hCGβcf (99/708) extractions were performed at 5 pmol/L concentration in stead of 0.5 pmol/L as for all other variants. Based on the recognition of both free and nicked hCGβ, together with the previously demonstrated hCGβcf reactivity, this antibody was classified as β_7 antibody.

Both mAbs ISOBM-403 and -435 targeted towards epitope β_1 were in fact the same antibody, and unwittingly served as internal controls. Their true identity was revealed after breaking the code and as can be seen in figure 2A-E, the results are highly concordant and display minimal variability.

hCG cross-contamination in hCGn (IRR 99/642)

LC-MS/MS of hCGn (1st IRR 99/642) confirmed a significant cross-contamination of approx. 20% by the intact i.e. non-nicked hCG molecule and to a smaller extent the hCGβ. This can be seen in figures 2C and 3 which show the differentiated hCG signals of the hCGn standard (99/642) for the various Abs. Thus, LC-MS/MS clearly differentiates which part of the signal actually originates from hCGn and which stems from the contamination with intact hCG. The chromatogram resulting from the analysis of the extraction of hCGn (99/642) by ISOBM-445 (epitope β_4-antibody) is shown in figure 4, that visualizes different peaks of the detected hCG variants. In addition to the two nicked hCG variants hCGn and hCGβn, intact hCG generates an unambiguous signal which confirms its presence as a cross-contamination. Since the affinity of

the anti epitope $\beta_{1\text{-}5}$ Abs towards the intact molecule seems to be slightly higher than the affinity towards the nicked variants, this method can only give a rough estimate of the cross-contamination content in hCGn (99/642). However, cross-contaminations in hCGn has previously been estimated by NIBSC to be approximately 23,5 % (17.5 % hCG plus 5.1 % hCGβ) (19).

It is apparent that there is a strong tendency for antibodies against intact hCG and hCG β-subunit to recognize both nicked heterodimer and nicked free β-subunit (figure 1C and 1D) (figure 1A) as well (figure 1B). For the evaluation of mAb performance towards the nicked variant containing the intact hCG contamination, this can be problematic for detection methods that generate a uniform signal that represents the sum of all captured variants. However, for the differentiating MS based detection this causes no problem.

c-mAbs

c-mAbs uniquely recognize the hCGαβ heterodimers but neither free subunit. Parts of their epitopes are located on beta-sheet loop 2 of hCGβ. Apart from sandwich assay compatibility a major criterion for the sub-classification is the recognition of hCGn, thus the influence of nicks between amino acids 44/45 and 47/48 in peptide loop 2 of assembled hCGβ. Although located in the same molecular region, epitopes $c_{1\text{-}3}$ can be either dependent on the intact loop (c_1 and c_2) or not (c_3). The value of LC-MS/MS data was particularly demonstrated when characterizing c-epitopes; by differentiating the signal of nicked and non-nicked hCG the MS signal permitted recognition of both nicked and non-nicked (cross-contamination) hCG from the nicked hCG standard (99/642) (figure 2C). This resulted in the specific sub-classification of the ISOBM-411 to recognize epitope c_2, which is not present on the nicked hCG. This sub-classification had previously been assumed, but was now verified by the MS data. The same specificity and

selectivity toward intact hCG was also demonstrated for the other c_2 Abs (ISOBM-425, -387, -436).

In the case of ISOBM-414, which has clearly been established as an Ab recognizing epitope c_1 by sandwich assays (reference), discordant results were observed by LC-MS/MS analyses. These showed that in addition to intact hCG, this Ab actually recognizes hCGn, and not just the intact hCG contamination in the nicked standard (Fig.2C). Thus it appears that ISOBM-414 is an exception to the rule that c_1 mAbs are specific for intact hCG (such as ISOBM-447). These data tell us that similar to what is observed for the epitope cluster of hCGβ loops 1 and 3 (epitopes β_2 to β_6), there seems to be some variability in epitopes of the c-domain, and thus a continuum of c-epitopes with slightly different Ab specificity patterns.

LC-MS/MS permitted the classification of mAbs which did not work in sandwich assays, such as the ISOBM-389. This mAb was known to be directed against a c-epitope, but its sub-classification was not established. As can be deduced from figure 1C the signal from hCGn proved to be derived from the hCG cross-contamination and this explained the apparent response to hCGn generated in DB-RIA (reference). Thus ISOBM-389 can't be directed against epitope c_3 like reference Ab ISOBM-446 since such Abs recognize both hCG and hCGn. It might however be an Ab directed against epitope c_2, such as mAb 411, or alternatively against epitope c_1, which is known to be specific for intact hCG, but the lack of hLH cross-reactivity contradicts such classification.

In general it appeared that c-mAbs recognizing hCGn did not differentiate between nicks 44/45 and 47/48 (Fig.3). Thus c_1 and c_2 epitopes are sensitive to either nick. Consequently neither epitope was dependent on just one of the nicks. This can most likely be extrapolated to be valid

for all nicking that might occur in the area of amino acids 44-48 of the hCGβ.that has been described (Ref)

Limitations of MS detection and discordant results

For two Abs, ISOBM-422 (epitope c_2) and ISOBM-386 (epitope β_7), no signals were generated in LC-MS/MS although the DB-RIA method provided appropriate signals. Furthermore it was observed that ISOBM-411 and -436, both directed against epitope $c_{2,}$ elicited minor signal responses. We have observed this phenomenon for other Abs as well when using the MS based targeted proteomics approach combined with Ab capture of proteins (data not published). This might be related to interaction between the capture Ab and the signature peptides for the target analytes, although antigen-Ab dissociation should have occurred due to the relatively harsh conditions (boiling, reducing conditions). The signature peptides of the hCG and hCGn as well as hCGβ and hCGβn is situated in the area of amino acids 44-60 corresponding to loop 2 (14). If the epitope was part of the signature peptides, binding of the Ab could have prevented trypsin cleavage and consequently detection of the signature peptide in LC-MS/MS.

A clear limitation of this MS based detection method is the inability to distinguish heterodimers from free subunits when these are detected through the same signature peptide. If hCG and hCGβ are mixed together, their differentiation will not be possible as they are both detected through the signature peptide βT5. This also applies for other variants; the nicked heterodimers cannot be distinguished from the nicked free subunits as long as the detection is based on the same signature peptide.

CONCLUDING REMARKS

In this report MS based evidence is provided regarding the epitope specificity of thirty hCG Abs from the 2nd ISOMB Workshop. In the majority of cases (25/30) the data confirmed their previously established epitope classification. For some Abs a more precise sub-classification was possible using the LC-MS/MS data. Furthermore, the high resolution has allowed us to confirm the presence of an hCG cross-contamination in the hCGn standard (1st IRR 99/642). This feature has had a notable impact, particularly for the evaluation of c-mAb specificity, since some of these recognize both hCG and hCGn whereas others recognize only hCG. Since cross-contamination of the hCGn standard by hCG does not compromise the MS detection, the unambiguous hCG-origin of c-mAb responses enables more precise sub-classification into c1, c2 or c3 epitope group for certain c-mAbs. The presented results provide complementary information on hCG mAb specificity and selectivity that can facilitate and strengthen the assumption of what is the best selection.

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LEGENDS TO FIGURES

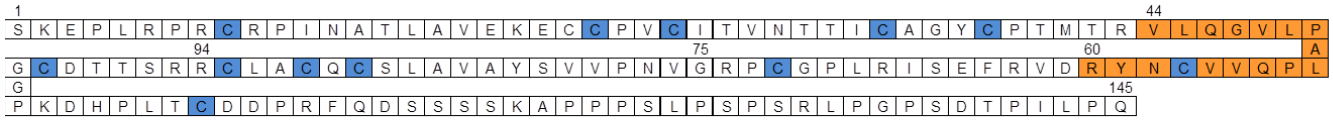
Figure 1. Schematic presentation of the hCGβ protein backbone of the various hCG molecules and their respective signature peptides including the signature peptide of hCGα. The cysteine residues which constitute the disulfide bonds are highlighted in blue. The hCG and hCGβ signature peptide (βT5) is marked in orange, the signature peptides of hCGn and hCGβn (nβT5' and nβT5) as well as that of hCGβcf (cfβT9) are marked in green. The signature peptide of hCGα (αT2) is marked in red.

Figure 2. Comparative classification of ISOBM-mAbs by DB-RIA and Immuno-LC-MS/MS. The results from classification of mAbs according to their main epitope specificities by DB-RIA are taken from the workshop report published in this issue of TumorBiology (ref.) The measured normalized MS signal intensity is represented by the ratio generated by the MS signal intensity of the detected hCG variant divided by that of the internal standard. The IRR antigens are: hCG (hCG 99/688), hCGn (hCGn 99/642), hCGβ (hCGβ99/650, hCGββ (hCGβn 99/692), hCGβcf (hCGβcf 99/708) and hCGα (hCGα 99/720).

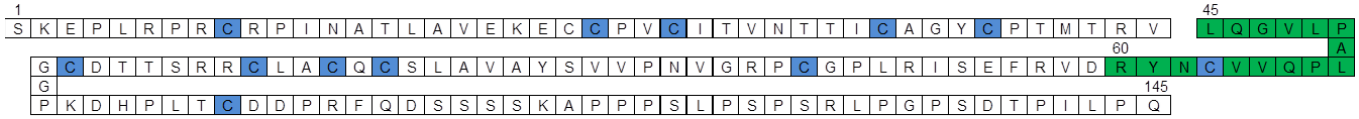
Figure 3 Mass spectrometric analysis of hCGn (99/642). The signal response detected for the two nicked hCG variants (44/47 and 47/48) has been differentiated. The signal of the hCG cross-contamination is marked in red, the signal of hCGn 44/45 is dark blue, and the signal of hCGn 47/48 is light blue.

Figure 4 LC-MS chromatogram from the analysis of the extraction of IRR 642 (hCGn) by ISOBM-445. Only the peaks representing the detected hCG variants are shown, at their respective retention times. All signal responses are normalized. A thorough explanation of MS

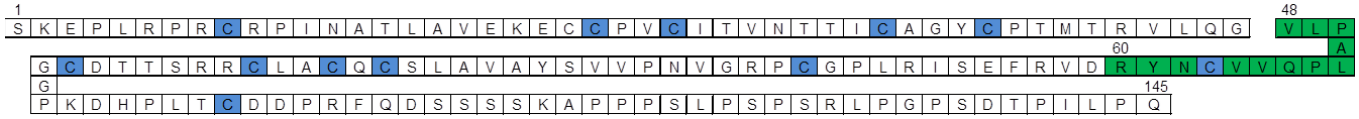
367 based analytical evidence for identification of the hCG variant identification is reported in a
368 previous study (14).
369
370



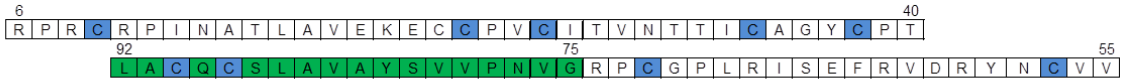
hCG and hCGβ: amino acid 1-145
Disulfide bonds: 9-57, 23-72, 26-110, 34-88, 38-90, 93-100
Signature peptide βT5: 44-60



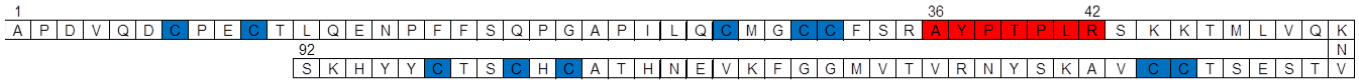
hCGn and hCGβn 44/45: amino acid 1-44, 45-145
Disulfide bonds: 9-57, 23-72, 26-110, 34-88, 38-90, 93-100
Signature peptide nβT5': 45-60



hCGn and hCGβn 47/48: amino acid 1-47, 48-145
Disulfide bonds: 9-57, 23-72, 26-110, 34-88, 38-90, 93-100
Signature peptide nβT5: 48-60



hCGβcf: amino acid 6-40, 55-92
Disulfide bonds: 9-57, 23-72, 34-88, 38-90
Signature peptide cfβT9: 75-92



hCGα: amino acid 1-92
Disulfide bonds: 7-28, 10-31, 32-60. 59-87, 82-84
αT2: 36-42

371

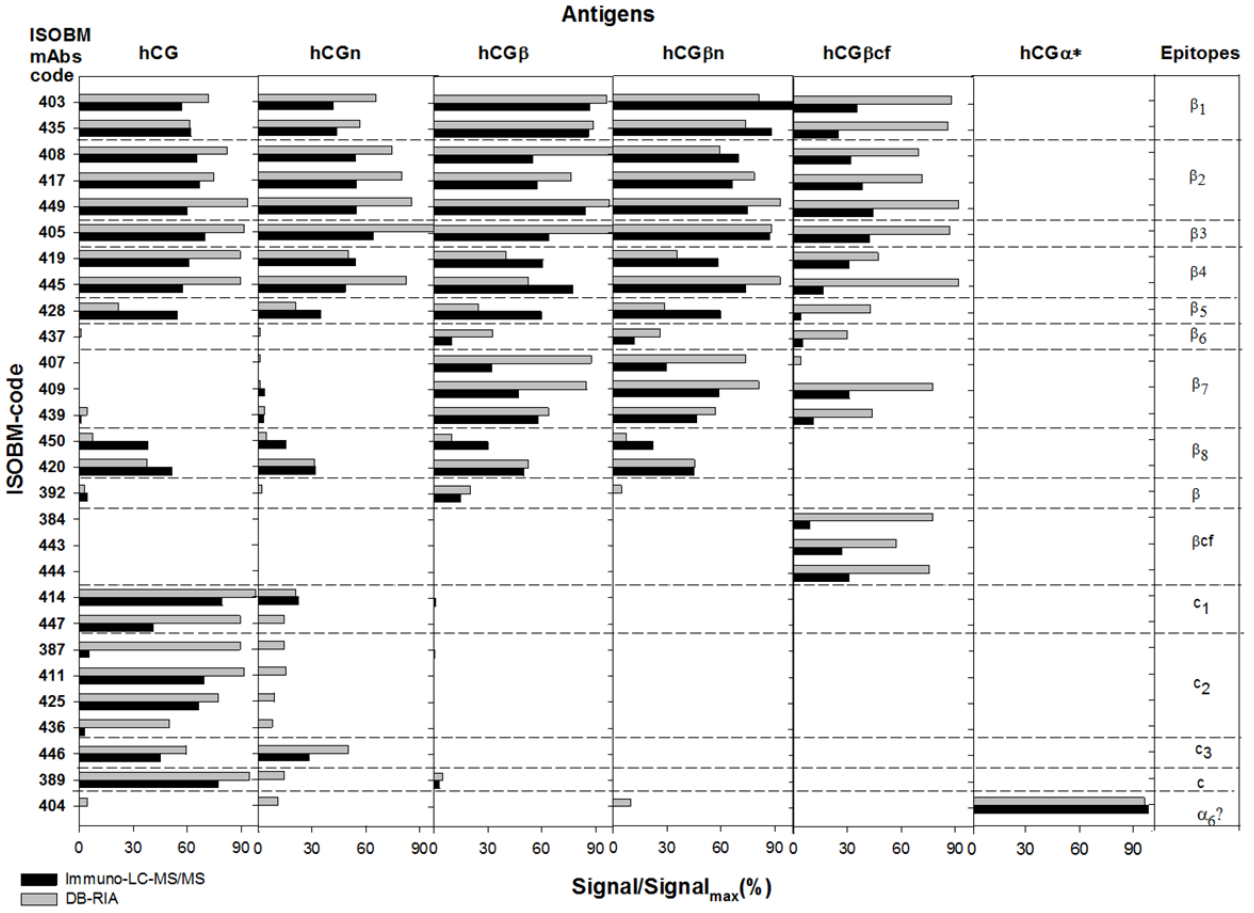

Antigens
ISOBM mAbs code
hCG
hCGn
hCGβ
hCGβn
hCGβcf
hCGα*
Epitopes
ISOBM-code
403
435
408
417
449
405
419
445
428
437
407
409
439
450
420
392
384
443
444
414
447
387
411
425
436
446
389
404
β1
β2
β3
β4
β5
β6
β7
β8
β
βcf
c1
c2
c3
c
α6?
0
30
60
90
Immuno-LC-MS/MS
DB-RIA
Signal/Signalmax(%)

Figure 2

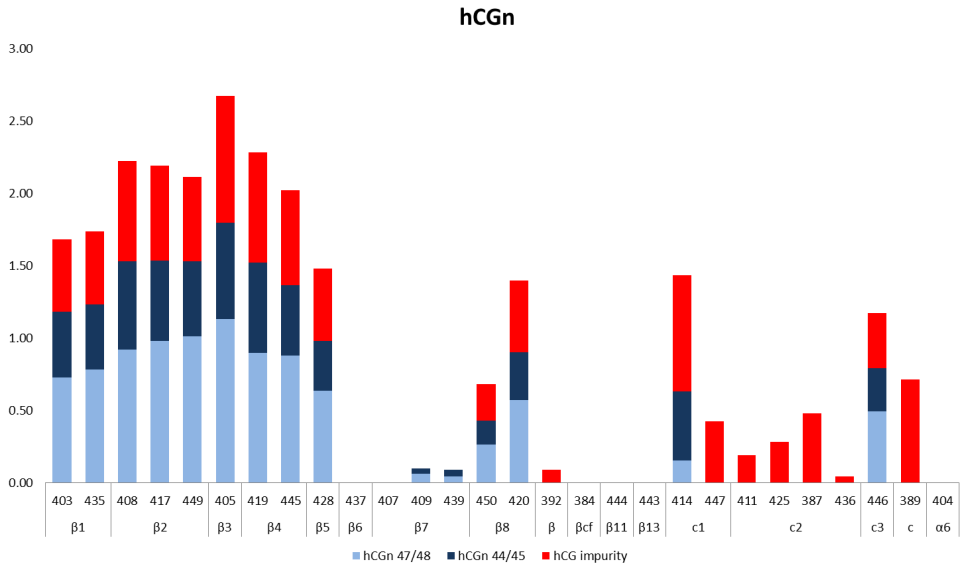

hCGn
3.00
2.50
2.00
1.50
1.00
0.50
0.00
403 435 408 417 449 405 419 445 428 437 407 409 439 450 420 392 384 444 443 414 447 411 425 387 436 446 389 404
β1 β2 β3 β4 β5 β6 β7 β8 β βcf β11 β13 c1 c2 c3 c α6
hCGn 47/48
hCGn 44/45
hCG impurity

Figure 3

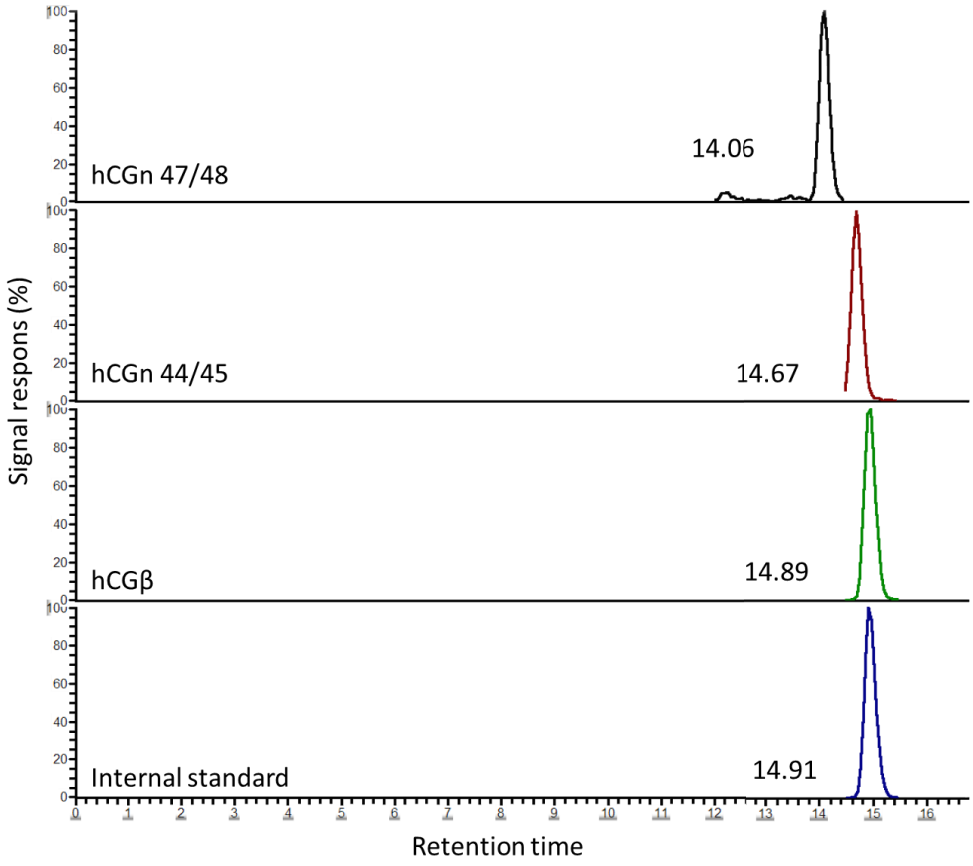

Signal respons (%)
hCGn 47/48
14.06
hCGn 44/45
14.67
hCGβ
14.89
Internal standard
14.91
Retention time

Figure 4